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SUNDAY 20 AUGUST, AFTERNOON

PLENARY LECTURES

S1. 2.40pm

Carbohydrate Receptor Recognition by Cholera and Related Bacterial Toxins

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Cholera toxin (CT) and the closely related heat-labile enterotoxin of *E. coli* (LT) are complex AB_5 protein assemblies. The enzymatic A subunit catalyses the ADP-ribosylation of $G_{s\alpha}$ which leads to deregulation of the adenylate cyclase system and eventually cholera and cholera-like disease. The B pentamer is responsible for recognition of the pentamer-saccharide head group of ganglioside G_{M1}, the receptor of CT and LT on the surface of the target epithelial cells of the intestinal system.

The recognition of the ganglioside G_{M1} by the CT B pentamer has been revealed crystallographically as well as the recognition of galactose, lactose, and galactose-galactosamine ('T-antigen') by the LT B-pentamer. Also mutants of CTB which have altered saccharide binding properties have been crystallographically characterized. A surprising result of these studies is the fact that a side chain of a residue of a B subunit of a neighbouring pentamer occupies the galactose binding site. Implications of this discovery for the design of cholera toxin antagonists will be discussed. The mode of binding of the B pentamer to the cell membrane and its consequence for the mode of cell entry of the A subunit will also be considered.

S2. 3.10pm

The Glycoprotein Hormones: The Functional Significance of Glycosylation

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Lutropin, thyrotropin, and a number of other glycoproteins bear unique oligosaccharides terminating with SO_4-4-GalNAc-β4GlcNAcβ. We have shown that a cluster of basic amino acids within two turns of an α-helix in the α-subunit are essential elements of a recognition determinant utilized by the GalNAc-transferase, which displays both peptide and oligosaccharide specificity. Structural features of the α-subunit essential for hormone dimerization, receptor binding, receptor activation, and GalNAc-transferase recognition are encoded within a sequence which is absolutely conserved from fish to mammals. Further, GalNAc-4-sulfotransferase and GalNAc-transferase are expressed in the pituitaries of all vertebrates, and a fish glycoprotein hormone bears sulfated oligosaccharides. Thus, the carbohydrate structure, like the peptide, is highly conserved. The sulfated structures are recognized by a hepatic endothelial cell receptor which serves to limit the circulatory half life of the hormone following release into the blood. The presence of these sulfated carbohydrates and their rapid clearance are essential for obtaining maximal hormone bioactivity *in vivo*. To assure that lutropin oligosaccharides are fully modified, GalNAc- and GalNAc-4-sulfotransferase levels *in vivo* are modulated in gonadotrophs in response to oestrogen in the same manner as their substrate lutropin.

S1. CONFORMATIONAL STRUCTURES OF GLYCOCONJUGATES

Supported by Lederle-Praxis Biologicals

Chairs: Hans Vliegenthart, David Bundle

S1. 4.00pm

Glycosylation, Recognition and Infectious Disease

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The infectivity, survival or pathogenicity of many parasites and pathogens involve oligosaccharides of either the invading organism or the host. An understanding of the role of the oligosaccharide at the molecular level is a first step towards the design of novel chemotherapeutic agents as candidate drug molecules. In particular, knowledge of the conformation of the relevant oligosaccharide both in the solution state and when bound to the protein receptor is ideally required for rational structure-based drug design. Recent work will be described aimed at solving such structures in solution using recent advances in high-resolution NMR spectroscopy.

S1. 4.30pm

Studies on the Glycan Structure of Glycoproteins

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In defining the structure of glycans of glycoproteins the complete primary structure of the glycans, the location of the glycosylation sites in the protein and the conformation of glycans have to be determined. Our work on the primary structures of glycans in a large array of glycoproteins has revealed that in many cases the microheterogeneity is formidable. For the location of glycosylation sites, it has recently been established that C-glycosylation of a specific tryptophan residue can occur in human RNAse U_s. With respect to the conformational studies on glycans of glycoproteins attention has focused on the pineapple stem bromelain and on a glycopeptide derived from this enzyme. A combination of simulations of molecular dynamics (MD) with NOESY ^{1}H-NMR spectroscopy was applied to

Manα6(Xylβ2)Manβ4GlcNAcβ4(Fucα3)GlcNAcβ-R

with R = peptide or protein.

Theoretical NOESY cross-peak intensities were calculated on the basis of models, generated by MD simulations. Several calculation methods were compared, which account differently for internal flexibility. These methods use generalized order parameters and/or apply an individual rotation correlation time for each monosaccharide residue. The Manα6Man and Fucα3GlcNAc linkages in the glycopeptide were found to be more flexible than the others. For the intact glycoprotein, the Fucα3GlcNAc and Manα6Man linkages were found to be altered in comparison to the glycopeptide. The findings may indicate an interation of the protein chain with the side of the N-glycan, where αFuc and αMan residues are present.

S1. 5.00pm

^{1}H and ^{13}C Chemical Shift Anisotropy Studies of Polymeric Carbohydrates: An Approach to Detecting Hydrogen Bonding Phenomena in Solution

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Sensitive pulse sequences were developed in order to determine geometry dependent ^{1}H and ^{13}C chemical shift anisotropy terms (CSA_g) of simple carbohydrates and carbohydrate polymers in solution. The methods are capable of monitoring CSA/DD cross-correlated relaxation for ca. 1 mM solutions at natural abundance. Measured spectral densities were quantitatively interpreted for sialic acid and its α-(2 → 8) linked homopolymer, colominic acid. The α and β cyclodextrins were also studied and their CSA values compared to α-methyl glucopyranoside.

Although the geometric factors could not be separated from anisotropy terms, this is the first report of CSA data on carbohydrate polymers and the values differ substantially from those of the corresponding monomers. The differences may be attributed to conformational changes, changes in hydrogen bonding interactions, and differences in OH rotamer populations. Application of these combined NMR methods provide a new parameter which may be sensitive to structural changes not detected with conventional NMR techniques.

S1. 5.20pm

Conformational Analysis of Oligosaccharides Employing Unrestrained MD Simulations: What Criteria are Appropriate for Comparing Experimental and Theoretical Data?

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NMR spectroscopy is an invaluable aid in deducing the conformational and dynamic properties oligosaccharides. However, NMR data alone are frequently insufficient to uniquely determine an oligosaccharides' conformation. We describe a complimentary computational approach. A 1 ns simulation of the fully solvated oligosaccharide $Man_9GlcNAc_2OH$, performed with the GLYCAM-93 parameter set and the AMBER force field is presented. The MD data are analysed in terms of average glycosidic torsion angles, as predicted by the isolated spin-pair approximation, and by nOe build-up curves computed from a full relaxation matrix analysis, averaged over the course of the simulation. Although nOe restraints were not employed during the simulation, close agreement between the computed and the experimentally-derived conformations was observed. The extent to which differences in absolute nOe intensities are

reflected in glycosidic torsion angles is examined. The roles played by explicit solvent-solute and intra-solute interactions are discussed.

S1. 5.40pm

Site-Specific and Complete Enzymatic Deglycosylation of the Native α-Subunit of Human Chorionic Gonadotropin

C. W. E. M. van Zuylen, T. de Beer[1], G. J. Rademaker[2], J. Haverkamp[2], J. E. Thomas-Oates[2], K. Hård[3], B. R. Leeflang[1,2], J. P. Kamerling[1], and J. F. G. Vliegenthart[1]

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Human chorionic gonadotropin (hCG) is a heterodimeric glycoprotein hormone. The α-subunit of hCG has two N-glycosylation sites, Asn78 and Asn52. Glycosylation at Asn52 has been shown to be involved in biological activity. NMR resonances of the carbohydrate moieties have been assigned [1]. A comparison of the structure of the native α-subunit with that of its site-specifically and completely deglycosylated forms can shed further light on how glycosylation influences activity.

Complete enzymatic deglycosylation of αhCG was achieved using endoglycosidase B. Full deglycosylation was shown using SDS-PAGE and 2D ^{1}H NMR spectroscopy. To our knowledge this is the first report of full deglycosylation of αhCG using non-denaturing conditions. In contrast, digestion of αhCG with PNGase F using non-denaturing conditions resulted in partial deglycosylation. 2D TOCSY spectra indicated that this deglycosylation is site-specific. FAB-MS results show that the site that has been specifically deglycosylated is Asn52. The selectivity of PNGase F for glycans attached to Asn52 of native αhCG suggests a higher level of steric hindrance at Asn78 than at Asn52. This is supported by the occurrence of ^{1}H-^{1}H NOEs between the GlcNAc-1 attached to Asn78 and several amino acids. Furthermore, NMR data suggest higher mobility for the glycan attached to Asn52 but reduced mobility for GlcNAc-1 of the oligosaccharide attached to Asn78.

1. de Beer T, van Zuylen CWEM, Hård K, Boelens R, Kaptein R, Kamerling JP, Vliegenthart JFG (1994) *FEBS Lett* **348**: 1–6.

S1. 6.00pm

Sialic Acid Defines the Conformational Epitope of the Capsular Polysaccharide of Type III Group B *Streptococcus*

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The capsular polysaccharide (CPS) of the type III group B *Streptococcus* (GBS) can be described as a conformational epitope because binding of its oligosaccharides (OS) to protective antibodies increases with the number of pentameric repeating units in the OS. Its sialic acid component is essential for the formation of the conformational epitope, but it is not directly involved in the binding to antibodies.

Interaction of sialic acid with the backbone of the PS was found to be important in defining the conformational epitope of the GBS III CPS. Conformational analysis using nuclear magnetic resonance was performed on a series of oligosaccharides of one to five repeating units of GBS III, desialylated OS and PS, oxidized PS and reduced PS. Conformational changes were gauged by chemical shift analysis, NOE studies, J(H,H) and J(C,H) coupling constants variations. Changes in mobility were examined by ^{13}C T_1 and T_2 measurements. Unrestrained molecular dynamics simulations with explicit water using the AMBER force field and the GLYCAM parameter set were used to assess conformational models.

S1 POSTERS

S1

Molecular Dynamics Simulations of Mucin-Type Multiply O-Linked Glycopeptides

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Mucins are highly extended glycoproteins whose properties are dominated by the presence of multiple O-linked oligosaccharide side chains. Earlier studies from our lab have shown the mucin carbohydrate are responsible for expanding the mucin chain dimensions by up to a factor of three compared to the nonglycosylated peptide core. To further understand the origins of the mucin's expanded conformation we have begun performing molecular dynamics calculations on a series of model mucin glycopeptides and their nonglycosylated derivatives. The glycopeptides being studied, $AAA\overset{*}{T}\overset{*}{T}\overset{*}{T}\overset{*}{T}\overset{*}{T}AAA$ and $AAA\overset{*}{T}P\overset{*}{T}P\overset{*}{T}AAA$ (where A = Ala, $\overset{*}{T}$ = α-GalNAC-O-Thr, and P = Pro), were chosen to model portions of the tandem repeat of the human Muc2 mucin and to determine the relative differences in peptide stiffness between the two motifs. Calculations were performed using the AMBER force field (as implemented by Biosym Tech. Inc.) using 14–15 Å nonbonded cutoffs. The glycopeptide was placed in a box of periodic boundary TIP3P water and calculations were performed for 200 to 400 ps. Differences in both end-to-end distances and the relative amplitude of motion are found for the various systems, with $AAA\overset{*}{T}\overset{*}{T}\overset{*}{T}\overset{*}{T}\overset{*}{T}AAA$ being the most rigid and its nonglycosylated derivative the least.

Supported by NIH grant RO1 DK39918.

S1

Solution Dynamics of the α-D-Idopyranose Pentaacetate Ring

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The dynamic behaviour of the pyranose ring of α-D-idopyranose pentaacetate in acetone has been characterized by line

shape analysis, T_1 and NOE measurements, and spectral density mapping. No evidence was observed for ring motion on timescales significantly shorter than 1 ns or longer than 1 ms. The forward and reverse activation energies for the two-state model interconversion between the $^4C_1$ and $^0S_2$ ring conformations has been determined to be 32 and 27 kJ mol^{-1} (at 273 K), respectively, from lineshape analysis. The corresponding exchange rate constants are 4.3×10^6 and 3.9×10^7 s^{-1}. These measurements represent the first experimentally determined kinetic data for idopyranose compounds.

S1

Favoured Conformations and Flexibility of Oligosaccharides: Application of Systematic Search and Genetic Algórithms in Parallelized HSEA-calculations

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Systematic conformational search was applied to study favoured conformations and flexibility of oligomannosides and blood group active oligosaccharides. The calculations were performed with a program which is a PVM (parallel virtual machine)-implementation of the classical HSEA-calculation [1]. The program uses energy filtering at the disaccharide level to exclude regions in conformational space with very high energies.

For Man3 and derivatives thereof a systematic search was performed in order to locate favoured conformations and to analyse the flexibility of the bonds of the glycosidic linkages. Different parameters proposed for the exo-anomeric effect [2, 3] were evaluated. For ABO- and Lewis active oligosaccharides similar calculations were applied to analyse the conformational restrictions imposed by fucose branches.

Despite the very significant speedup achieved using filtering and parallelization, systematic search is still prohibitively time consuming for larger oligosaccharides (>6 residues). For conformational search on large oligosaccharides a genetic algorithm [4] (GA) has been implemented with multi-level parallelism. This GA-method has been used to locate favoured conformations of Man3 and Man5. Comparisons between the GA-method and the systematic search in terms of speed and success in detecting low energy conformations will be reported.

1. Lew T, Nyholm PG, Carver J (1995) ACS Proc. Anaheim, April 1995.
2. Tvaroska I, Carver J (1994) *J Phys Chem* **98**: 9477–9485.
3. Wiberg KB, Marquez MJ (1989) *J Am Chem Soc* **111**: 4821–4828.
4. Judson RS *et al*. (1993) *J Comp Chem* **14**: 1385–1395; 1407–1414.

S1

Peptide-Carbohydrate Interactions in a Glycopeptide from Human Serotransferrin

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Intramolecular peptide-carbohydrate interactions are potentially important factors in determining glycoprotein structure and function. In this context, we are studying a glycopeptide consisting of a 21 amino acid polypeptide (G^{603}-R^{623}) with a sialylated diantennary oligosaccharide attached to N^{611}, obtained by tryptic digestion of human serotransferrin [1]. Having assigned the complete ^{1}H-NMR spectrum of the glycopeptide in H_2O, we primarily use two-dimensional ^{1}H-^{1}H nuclear Overhauser effect spectroscopy (NOESY) as the experimental tool to probe the conformation of the glycopeptide in aqueous solution. We have observed and identified over 280 contacts in the NOESY spectra of the glycopeptide, including a number of remote contacts between sugar and amino acid protons. We have constructed a model structure for the glycopeptide based on distance geometry calculations, restrained by the experimental NOE contacts. Molecular dynamics simulations for the glycopeptide are in progress. We will evaluate our results in the light of peptide-carbohydrate interactions observed by others in X-ray structures of glycoproteins.

Supported by the NIH Resource Center Grant for Biomedical Complex Carbohydrates (P41-RR05351).

1. Fu D, van Halbeek H (1992) *Anal Biochem* **206**: 53–63.

S1

Carbon-13 NMR Studies of a Human Goblet Cell Line Mucin

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^{13}C-NMR spectroscopy is being used to study the structure and dynamics of native and sequentially deglycosylated mucin produced by the HT29-CL16E human goblet cell line. ^{13}C-NMR spectra of the mucin reveal considerably less carbohydrate side chain structural heterogeneity compared to most human mucins, with the [α-NeuNAc(2-3)β-Gal(1-3)]- [α-NeuNAc(2-3)β-Gal(1-4)β-GlcNAc(1-6)]-α-GalNAc-O-Ser/Thr hexasaccharide being essentially the only side chain. Relaxation time measurements on native mucin suggest the terminal NeuNAc and Gal residues have similar mobilities which are somewhat elevated compared to the 2-6 linked NeuNAc residues in the shorter side chains of porcine or ovine submaxillary mucin. To monitor the peptide core and peptide linked GalNAc dynamics, whose resonances are difficult to observe in native mucin, we have determined conditions for the optimal incorporation of ^{13}C-labelled proline, glycine and glucosamine into the high molecular weight glycoconjugates and mucins produced by these cells. The ^{13}C NMR spectrum of labelled mucin is consistent with the expected metabolic pathways for the added substrates, thus providing several additional labelled positions for relaxation time studies.

Supported by NIH grants PO DK27651 and RO1 DK39918.

S1

Determination Of The Stereochemistry Of Alkyl Chains In Cyclic Acetal Of Carbohydrate By NMR

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Glycosphingolipids are present in most mammalian tissues and

play important roles in cellular recognition and transmembrane signalling. Sphingosine, N,N-dimethylsphingosine, N,N,N-trimethylsphingosine, and lysosphingolipids such as lyso-GM3 and psychosine have been identified as potent inhibitors of protein kinase C. Two novel lysosphingolipids, Plasmalopsychosine A and Plasmalopsychosine B, have been isolated from the white matter of human brain. A cyclic acetal of galactose with a C_{16} aliphatic aldehyde is a part of both of these structures and is believed to be responsible for the unique biological properties shown by these novel lysosphingolipids. We have reported the total synthesis of one isomer of Plasmalopsychopsine A, which is a 3,4-cyclic acetal, and one isomer of Plasmalopsychosine B, which is a 4,6-cyclic acetal, and have shown their identity with the compounds isolated from the natural source. In order to compare the general structural features of stereoisomeric C_{16} cyclic acetals of galactose, we synthesized C_{16} cyclic acetals of methyl β-D-galactopyranoside. Following peracetylation, it was possible to separate all four stereoisomeric cyclic acetal structures. The structural differences of these four isomers were determined by the use of NMR spectroscopy.

S2. GLYCOSYLATION AFFECTING PROTEIN STRUCTURE AND FUNCTION

Chairs: Minoru Fukuda, R. Colin Hughes

S2. 4.00pm

A Newly Cloned Polysialyltransferase Forms the Embryonic Form of N-CAM that Facilitates Neurite Outgrowth

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Polysialic acid is a developmentally regulated post-translation modification taking place in both glycoproteins and glycolipids. Recently we have cloned polysialyltransferases that form polysialic acid in glycoproteins (PST) and in glycolipids (GT3ST), respectively. The newly cloned polysialyltransferases belong to the sialyltransferase gene family and have homology with each other. However, PST catalyses polysialylation only in glycoproteins and contains distinct basic amino acid clusters. The expression of polysialic acid detected by antibody 735 correlates well with the expression of PST mRNA in various fetal and adult human tissues.

In order to determine the roles of polysialic acid in neural development, we tested the way in which PST expression in subsaturn cells influences neurite outgrowth. We thus expressed N-CAM alone or N-CAM and polysialic acid on substrate HeLa cells. Neurons from both dorsal root ganglion and ventral portion of spinal cord, derived from chicken embryos, exhibited longer neurites and more branching on HeLa cells expressing polysialic acid and N-CAM than on HeLa cells expressing N-CAM alone. These results indicate that the cloned PST forms polysialylated, embryonic N-CAM, which is critical for the plasticity of neural cells. Similar studies are in progress to address the roles of polysialylated glycolipids. The cloned PST and possibly GT3ST are thus powerful tools to dissect the intricate and complex processes of cell-cell interaction during development.

Supported by CA33895, DK37016 and HD25938.

S2. 4.25pm

The Role of Glycosylation in Regulating Gonadotropin Free Alpha and Free Beta Subunit Combination in Early Pregnancy

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The family of glycoprotein hormones consists of a common α subunit combined with one of four different β subunits resulting in dimer hormones with different biological specificities. The presence or absense of a glycan at each of the 3–4 N-linked glycosylation sites of the various hormones has been shown to have dramatic impact on secretion and/or receptor binding and signal transduction. The recent discovery that an α subunit alone (free α subunnit) stimulates prolactin secretion from decidual cells establishes free α as a hormone with activity that is independent of dimer hormones. During early pregnancy, free α and free hCG-β subunits coexist in the same placental compartment in concentrations sufficiently high that combination would be expected to occur. We evaluated these free subunits for their ability to combine with their complementary subunit obtained from dissociated hCG. The placental free β was able to combine with hCG-α to form intact hCG. In contrast, the placental free α did not combine with hCG-β to form hCG. However, after removal of the N-linked glycans from free α by treatment with PNGase F, most of the free α subunits were able to combine with hCG-β. These data indicate that N-linked glycan(s) on placental free α prevent the molecule from combining with available and combinable free β subunits that coexist in the same gestational compartment, thus ensuring availability of both free subunits. This impact of glycans on the status of the free subunits further extends the sphere of influence that glycosylation has on the function and physiology of the glycoprotein hormone family.

S2. 4.50pm

'Brain-Type' Glycosylation of Intrathecally Synthesized Secretory Proteins in Human Cerebrospinal Fluid: Comparison to Their Recombinant Counterparts and the Natural Human Serum Glycoforms

Andrea Hoffmann, Eckart Grabenhorst, Manfred Nimtz and *Harald S. Conradt*
Department of Gene Regulation and Differentiation, GBF, Mascheroder Wegl, D-38124 Braunschweig, Germany.

Some 80% of the total proteins contained in human cerebrospinal fluid (CSF) are derived from serum via transfer across the blood brain barrier (BBB) by unknown mechanisms. The remainder is synthesized locally. We have isolated the human 'β-trace' protein (β-TP) from pooled human CSFs and have

characterized the N linked oligosaccharides of this polypeptide which constitutively secreted by plexus chorioidae tissue [1]. Using methylation analysis, HPAE-PAD mapping and LSI-MS, N-linked complex-type glycan structures of β-TP were shown to contain proximal α1-6 fucose, Lewis[x] and sialyl Lewis[x] motifs, bisecting GlcNAc and were undersialylated (α2-3 and α2-6-linked NeuAc). Fifty per cent of truncated (asialo-agalacto) chains were found. An unsialylated form of transferrin (asialo-Trf [1] was isolated from human CSF, which exhibited the same carbohydrate structural features as detected in β-TP described above. Such structures have never before been detected in human transferrins; asialo-Trf from CSF is obviously of local origin. A fully sialylated serum-type transferrin form, however, isolated from the same source, lacked these structural characteristics of 'brain-type' N-glycosylated polypeptides and is presumably derived from serum via transport across the BBB. Significant glycosidase activity was not detected in human CSF. Molecular cloning of the human β-TP cDNA following expression and purification of recombinant β-TP from BHK-21 cells revealed biantennary N-glycan structures with almost fully α2-3 sialylated intact lactosamine antennae. These results support the tissue-specific '*brain-type glycosylation*' of locally synthesized glycoproteins secreted in human CSF. The purified recombinant proteins expressed from BHK-21 and from insect cell lines are presently being used for *in vivo* studies in rat.

1. Hoffmann *et al.* (1993, 1994) *J. Neurochem* **61**: 451–56 and **63**: 2185–96).
2. (Hoffmann *et al.* (1995) *FEBS Lett* **359**: 164–68).

S2. 5.10pm

Glycosylation and Antibody Function

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IgG molecules are glycosylated in C_H2 at Asn 297; the N-linked carbohydrates attached there have been shown to contribute to antibody stability and various effector functions. The carbohydrate characteristics of the IgG constant (Fc) region is a complex biantennary structure. Alterations in this oligosaccharide, specifically reduced levels of galactosylation, have been associated with human diseases such as rheumatoid arthritis. To study the effects of altered glycosylation on antibody effector functions, we have used gene transfection techniques to produce mouse-human chimeric IgG1 antibodies in Chinese hamster ovary (CHO) cells with different mutations in the biosynthetic pathway for N-linked glycosylation. These include cell lines deficient in terminal processing steps such as galactosylation and sialylation, as well as a cell line incapable of processing the high-mannose intermediate (Lecl). Antibodies were also produced in wild-type Chinese hamster ovary cells to confirm that the proteins would be expressed and secreted as functional antibodies. All the CHO cell lines secreted antibodies that retained antigen specificity. The Lecl-produced antibodies have been shown to be deficient in complement activation, have reduced but significant affinity for FcγRI receptors, and a shorter *in vivo* half life than antibodies produced in either myeloma or wild type CHO cells. IgG1 antibodies have been produced in additional CHO cell lines with specific glycosylation defects to evaluate the contribution of carbohydrate structure to effector functions.

S2. 5.30pm

N-Glycans Modulate Thyroid Hormonogenesis at the N-Terminal Domain of Thyroglobulin

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Thyroglobulin (Tg), the major glycoprotein (2×330 kDa, about 10% carbohydrates) of the thyroid gland, is the substrate of thyroid hormones (T4 and T3) biosynthesis which occurs at the apical membrane and involves iodination of Tyr residues into iodotyrosines and coupling of few of them into hormones. It is known that Tg N-glycans play a role in recycling poorly iodinated Tg through a GlcNAc receptor. Therefore, poorly iodinated Tg is not degraded after endocytosis but, rather, recycled via the Golgi apparatus to the colloid in order to complete its iodination and, ultimately, proceed to hormone formation. In the present study, we show that the presence and the structure of N-glycans are also directly involved in hormone synthesis.

The N-terminal domain of human Tg (NTD, Asn1-Met171, N-glycosylated at Asn57 and Asn91) contains the preferential T4-forming site at Tyr5 and is also able to form T4 *in vitro*. NTDs were purified after CNBr treatment from Tg preparations with low and mild iodine content *in vivo*. Using affinity chromatography on ConA- and RCA120-Sepharose columns, the NTDs were separated in five isoforms according to the type and the degree of N-glycosylation and were tested for T4 content. Our results showed that: 1) *in vivo* as well as *in vitro*, unglycosylated isoforms did not form hormone, while fully or partially (at Asn91) glycosylated ones did; 2) the presence of high mannose type structures enhanced the hormone content; 3) desialylation did not affect *in vitro* hormone synthesis.

In conclusion: 1) thyrotropin, which modulates the number and the nature of the oligosaccharide side chains born by Tg could also modulate the formation of T4 residues through the N-glycosylation process; 2) a defect in hormonosynthesis may derive from abnormal glycosylation of the NTD; 3) domain, rather than whole molecule, should be considered when ascribing a role to N-glycans.

S2. 5.45pm

Inhibition of Human HT-29 Colon Carcinoma Cell Adhesion and Homotypic Aggregation by a 4-Fluoro-Glucosamine Analog

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2-Acetamido-1,3, 6-tri-*O*-acetyl-4-deoxy-4-fluoro-α-D-glucopyranose (4-F-GlcNAc) was found to inhibit the biosynthesis of human HT-29 colon carcinoma cell glycoconjugates as measured by the incorporation of labelled sugar precursors: [^{3}H]GlcNH$_2$, [^{3}H]Fuc and [^{3}H]Gal. This inhibition led to decreased glycosylation of cell surface glycoproteins including lysosomal associated membrane proteins (LAMPS) 1 and 2, and carcinoembryonic antigen (CEA). These heavily glycosylated glycoproteins participate in adhesion and homotypic aggregation of HT-29 cells by providing ligands for specific lectins. Treatment of HT-29 cells with 4-F-GlcNAc resulted in decreased expression of sialyl-Le[x] and sialyl-Le[a], the cell surface

ligands for E-selectin. The aberrant glycosylation of cell surface glycoproteins resulted in: 1) decreased E-selectin mediated adhesion of human colonic HT-29 cells to endothelial cells; 2) impaired adhesion of HT-29 cells to β-galactoside binding lectin, galaptin; and 3) reduced ability to form homotypic aggregates. These results strongly suggest that modification of cell surface lectin receptors can alter tumour cell adhesion and recognition, and that these surface receptors may act as suitable targets for therapeutic exploitation.

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S2. 6.00pm

Site-directed Mutagenesis of Rat Leukemia Inhibitory Factor

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Leukaemia inhibitory factor (LIF) is a pleiotropic growth factor active in diverse cell systems in both the adult and the embryo. LIF which is expressed in various tissues and cells, has a molecular weight of 40–70 kDa. The molecular weight of the peptide portion is deduced to be 22 kDa from the nucleotide sequence of cDNA. This suggests that LIF is heavily glycosylated, although its glycosylation site and biological significance are still unknown. Therefore, we tried to produce the recombinant proteins for analysis of the N-glycosylation of LIF by site-directed mutagenesis. The cDNA of LIF was prepared by PCR from the cDNA library of rat placenta. There were seven pos
sible N-glycosylation sites at position (9, 34, 63, 73, 96, 116 and 150). Among them, the position of six aspargine residues at 9th, 34th, 63rd, 73rd, 96th, and 116th were conserved among animals. Point-mutated LIFs, which change one of these possible N-glycosyl residues, were produced in a SRα-COS 7 cell system by substitution of each aspargine residue for glutamine residue. Non-glycosylated LIF was generated by the E. coli-GST fusion system. The molecular weight of each mutant LIF was bigger than non-glycosylated one, indicating that native LIF possesses several N-linked sugar chains, which should be biologically important.

S2 POSTERS

S2

Probing the Active Site of Oligosaccharyl Transferase from Pig Liver with Synthetic Peptides as Tools

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Oligosaccharyl transferase (OST) catalyses the transfer of oligosaccharides from Dol-PP on to specific asparagine residues, provided that these are part of the sequence motif Asn-Xaa-Thr/Ser/Cys. We have purified the enzyme from pig liver [1] and studied its binding and catalytic properties using peptide analogues derived from *N*-benzoyl-Asn-Gly-Thr-$NHCH_3$ by replacing either Asn or Thr by amino acids differing in size, polarity, stereochemistry and ionic properties. Binding and acceptor studies showed that substitution of Asn or Thr by amino acids with bulky side chains (cysteinsulfonamide, Asp-β-methylester, Asp-β-hydroxamic acid for Asn; threo-β-hydroxy-asparagine, phenylserine for Thr) impaired recognition by OST. Also a threo-configuration at the β-C-atom of Thr was found to be essential for binding, pointing to a high degree of specificity of OST for the natural substrate. Reduction of the Asn-β-amide to an alkyl amine yielded a derivative which, though not glycosylated, was strongly inhibitory (K_i = 140 μM), due to ion pair formation with an anionic base at the active site. Substitution of the Thr-β-hydroxy by a β-amino group yielded a derivative with acceptor properties, indicating that the amino group can mimic the function of the hydroxy group during transglycosylation. The data are discussed in terms of the catalytic mechanism of OST and the role of the Thr side chain in the catalytic process.

1. Bause *et al*. (1995) *Eur J Biochem*: in press.

S2

Synthesis of Oligosaccharide Protein Conjugate With Intersecting *N*-Acetyl-glucosamine as Blood Group Determinants

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2-Phthalimido, 2-deoxy-β-glucopyranosyl moiety is one of the building blocks of several glycoproteins and lipopolysaccharides. Oligosaccharides containing a *N*-acetylglucosamine unit are important components of the human blood group system. They have been found to be antigenic determinants. Such oligosaccharide structures which lead to human ABH and Lewis antigenic determinants can occur as linear or branched chains and these di and tri-saccharide units, chemically synthesized as glycosides contain sequences unique to asparagine-linked carbohydrate chains of pituitary glycoprotein hormones. The glycoproteins contain an invariant oligosaccharide core structure and variation is provided by substitution with mono or oligosaccharide groups at different positions of this core structure which carries the antigenic specificity. In this context, we have synthesized oligosaccharides using different regio- and stereoselective reactions. Derivatives of Allyl glycosides of *N*-acetylglucosamine as glycosyl acceptors and different active glycosyl donors of D-galactose and L-fucose have been prepared. The mono- and disaccharide thus prepared will be conjugated to a high molecular weight matrix through the Allyl linker arm and thereby provide antigens for specific antibody production.

S2

Development of a Colon Tumor Metastasis Model in Nude Mice to Assess the Antimetastatic Activity of Sugar Analogues using Magnetic Resonance Imaging

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Adherence of human colon carcinoma cells is partially mediated by interactions between cell surface glycoconjugates and lectins,

while aggregation of these cells involves homophilic adhesions between glycosylated cell surface molecules. To alter these functions, 2-acetamido-1,3,6-tri-O-acetyl-4-deoxy-4-fluoro-α-D-glucopyranose (4-F-GlcNAc) was synthesized, and its effect on the function of cell surface glycoconjugates in human colon carcinoma HT-29 cells was evaluated. 4-F-GlcNAc was found to affect the biosynthesis of HT-29 cellular glycoconjugates, leading to decreased glycosylation of cell surface lysosomal associated membrane protein (Lamp) 1 and 2, carcinoembryonic antigen (CEA), and sialyl-Le^x and sialyl-Le^a. The aberrant glycosylation of HT-29 cell surface moieties resulted in: (1) impaired adhesion of cells to β-galactoside binding lectin, galectin-1; (2) decreased E-selectin mediated adhesion of HT-29 cells to endothelial cells; and (3) reduced ability of HT-29 cells to form homotypic aggregates. These results strongly suggest that modification of cell surface lectin receptors can alter tumor cell adhesion and recognition, and that these surface receptors may act as suitable targets for therapeutic exploitation. To assess the therapeutic efficacy of 4-F-GlcNAc, magnetic resonance (MR) imaging will be used to monitor, serially, inhibition of the formation of hepatic metastases in nude athymic mice following intrasplenic human colon tumor xenograft implantation.

S2

The Immunoreactivity and Function of the Carbohydrate Moieties of Cobra Venom Factor

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Cobra venom factor (CVF) is a nontoxic glycoprotein in cobra venom. CVF can cause exhaustive complement activation resulting in a massive build up of membrane attaching complexes. Based on this unique property, CVF has been studied for selective cell killing. Previous studies have shown that CVF immobilized to PVDF membranes reacts strongly with naturally occurring human anti-α-Gal antibodies. This immunoreactivity can be abolished by deglycosylation or treatment with α-galactosidase. However, it was not evident whether non-denatured, soluble CVF also binds anti-α-Gal antibodies. In the present study, soluble CVF inhibited effectively the binding of anti-α-Gal antibodies to CVF-coated microtitre plates. De-α-galactosylated and deglycosylated CVF showed very little inhibition. The data indicated that the anti-α-Gal antibodies can bind to CVF even in its native conformation. Modification of the terminal α-galactosyl residues of CVF by treatment with galactose oxidase and blocking of the generated aldehyde groups with various alkanoic hydrazides abolished the anti-α-Gal antibody binding but not the activity of CVF. The effect of carbohydrate moieties on the rate of clearance of CVF from circulation in mice was also investigated. CVF, de-α-galactosylated CVF and deglycosylated CVF were radioiodinated and their plasma clearance studied. The rate of clearance was not altered on removal of α-galactosyl residues or upon deglycosylation suggesting that the density of terminal galactosyl residues in CVF is not sufficient to permit its binding to hepatic asialoglycoprotein receptors.

S2

Functional Improvements in β-Lactoglobulin by Conjugating with Carboxymethyl Dextran

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Bovine β-lactoglobulin (β-LG) was conjugated with carboxymethyl dextran (CMD) to improve the protein function by using water-soluble carbodiimide. Two β-LG-CMD conjugates (Conj. 10A and Conj. 10B) could be prepared. The molar ratio of β-LG to CMD in the conjugates was 7:2 (Conj. 10A) and 1:1 (Conj. 10B). The isoelectric point of each conjugate was 4.7–4.8, which is lower than that of β-LG. Spectroscopic studies suggested that the conformation around Trp had not changed in each conjugate but α-helix content of Conj. 10A had markedly decreased compared with that of β-LG. Structural analyses with monoclonal antibodies indicated the conformational change of 125Thr-135Lys (α-helix) in Conj. 10A and of 15Val-29Ile (β-sheet) in Conj. 10B. The denaturation temperature of each conjugate was about 89 °C, which is much higher than that of native β-LG. Each conjugate maintained retinol-binding activity as strongly as that of β-LG. By conjugating with CMD, the emulsifying ability of β-LG was greatly enhanced in the neutral and acidic pH range and in the presence of NaCl below 0.2 M. After heating at constant temperature (40–80 °C) for 10 min, the emulsifying ability of the β-LG-CMD conjugates was higher than that of β-LG under all heating conditions. Anti-β-LG antibody response was lowered by conjugation with CMD. This mechanism is discussed. We think that the conjugation with acidic polysaccharide is effective for the functional improvement of β-LG.

S2

Characterization and Cloning of Glucosidase I from Human Brain

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Glucosidase I, the first enzyme in the N-linked oligosaccharide pathway, cleaves with high specificity the distal α1,2-linked glucose residue in the Glc_3-Man_9-$GlcNAc_2$ precursor oligosaccharide. The enzyme has been purified from pig liver crude microsomes by affinity chromatography and poly(ethylene glycol) precipitation [1]. The purified glucosidase I has a molecular mass of 95 kDa. Susceptibility to endo H indicates that the enzyme is N-glycosylated, carrying a single oligosaccharide chain of the high mannose type. The enzyme has a pH optimum close to 6.4 and is strongly inhibited by 1-deoxynojirimycin dNM ($K_i = 3$ μM) and its N-methylated derivatives (*N*-methyl-dNM, $K_i = 0.3$ μM; *N,N*-dimethyl-dNM, $K_i = 0.4$ μM). On the basis of tryptic peptides obtained from the pig liver enzyme, degenerate oligonucleotides in both sense and antisense orientation were derived and a cDNA probe was synthesized by PCR using a porcine firststrand cDNA as the template. An 800 bp cDNA fragment was amplified encoding the amino acid sequence of several tryptic peptides in one ORF. Screening of a human hippocampus cDNA library with this probe resulted in the isolation of several glucosidase I-specific clones. The sequence of these clones covers 1632 bp, including the sequence of the screening probe and encoding the amino acid sequence of

twelve tryptic peptides. The 1632 pb fragment represents about 57% of the glucosidase I protein.

1. Bause *et al.* (1989) *Eur J Biochem* **183**: 661–69.

S2

Characterization of Placental β1,4-Galactosyltransferase with Two-Dimensional Electrophoresis and Carbohydrate Analysis

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UDP-Gal:GlcNAc β1,4-galactosyltransferase (GalT) was purified from a Triton X-100 extract of human full-term placenta by affinity chromatography on UDP-hexanolamine and α-lact-albumin agaroses. On 2D-PAGE, the 10000-fold purified enzyme gave multiple bands with a pI range of 5.1–6.7 and M_r of 43000–49000. The slightly upward-sweeping arch of spots indicated that the charge heterogeneity was due to sialic acid residues, and 2D-analysis of the neuraminidase-treated protein supported this view. In addition to lower molecular weight species, polypeptides with M_r of 53000–54000 were found in the neutral pH area. These bands obviously represented the full-length, membrane-bound form of the placental GalT; so far, human GalT has been isolated only in soluble form from various body fluids. Both carbohydrate analysis and lectin binding studies suggested the presence of short O-glycans and N-glycans of the complex type; no high-mannose or hybrid glycans were detected. Most if not all sialic acid residues were α2-3 linked. Thus, the placental GalT clearly differs from the soluble milk enzyme (M_r = 55000), which has extremely heterogeneous and complex O-glycosidic chains [1].

1. Amano *et al.* (1991) *J Biol Chem* **266**: 11461–77.

S2

Human Transferrin Receptor Oligosaccharides

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The human transferrin receptor (TfR) has three N-linked and one O-linked oligosaccharides. Using a combination of site-directed mutagenesis, carbohydrate and protein chemistry and mass spectroscopy we have characterized the structure of the N-linked oligosaccharides and mapped their locations. Following digestion of purified TfR with trypsin (3 × 1 h at 1:100 w/w) individual peptides are obtained via RP-HPLC. Peptides are assayed for monosaccharides by strong acid hydrolysis and HPAE-PAD. Peptides containing carbohydrate are subjected to gas-phase sequencing to identify the specific Asn residue and the oligosaccharides analysed by MALDI-TOF, HPAE-PAD, glycosidase digestions and Biogel chromatography. The oligosaccharide found at Asn-727 is critical for the proper folding, trafficking and function of the TfR; it contains a series of high mannose structures, some of which are phosphorylated. The glycopeptide containing the site at Asn-317 has been refractory to analysis and has only recently been obtained by a short (30 min) tryptic digestion, followed by V-8 protease digestion of a glycopeptide beginning at Val-268. The monosaccharide composition consists of Man, Gal and GlcNAc in a ratio suggesting a mixture of high mannose and complex or hybrid structures. This is consistent with endo H digestions of TfR from mouse 3T3 cells transfected with cDNA.

The oligosaccharides at Asn-251 are of the complex type and judging by HPAE analysis are triantennary and trisialylated. The O-linked oligosaccharide at Thr-104 appears to influence the proteolytic sensitivity of Arg-100, which generates the soluble, serum form of the receptor.

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S2

SV40 Large T Antigen Subpopulations are Differentially Glycosylated

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SV40 large T antigen plays as essential role in the infection cycle of the virus. T antigen has multiple functions including binding of p53, altering transcription patterns in host cells and replicating of viral DNA. T antigen has been reported to bear multiple post-translational modifications such as phosphorylation and glycosylation. Several workers have reported glycosylation of T antigen, but no known function for the modification, or agreement on a sugar structure has been reached. We have demonstrated the presence of O-linked *N*-acetylglucosamine (O-GlcNAc) on T antigen by metabolic labelling of SV40 infected cells with [^{3}H]-glucosamine, β-elimination of the radiolabelled T antigen releases [^{3}H]-glucosaminitol. β1,4 galactosyl transferase labelling of T antigen further confirms the presence of O-GlcNAc. We have found no evidence for the presence of other sugars on T antigen. Interestingly, the fraction of T antigen tightly associated with chromatin seems to be more highly glycosylated that the bulk T antigen. We are currently attempting to identify sites of glycosylation. In addition T antigen overproduced in a baculovirus system is not efficiently glycosylated. It has been shown that T antigen produced in this system is heavily phosphorylated, and we are examining the possibility of competition between O-GlcNAc and phosphate modification at the same sites. These studies should help us to understand better the function of O-GlcNAc on this important protein.

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S2

Changes in Biological Functions of Heterologous Proteins Expressed in the Methylotrophic Yeast *Pichia Pastoris*

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The selection of an appropriate host for heterologous expression of a given protein should not be limited only to the protein yield and localization within or without the cell. It should also be made with the biological function of the final product in mind, not only *in vitro* but *in vivo* as well. Heterologous protein glycosylation emerges as a crucial point to understand the role of oligosaccharide residues on its biological functions.

We have studied several proteins cloned and expressed in very high yield in the methylotrophic yeast *Pichia Pastoris* in terms of their glycosylation pattern and their relation with some

biological and biochemical characteristics. The most interesting result was that some of the industrial enzymes we analysed showed an enhanced thermostability with respect to the native enzyme without any change in enzymatic activity. This includes sucrose invertase from yeast, α-amylase from bacillus and dextranase from fungus.

Some other *in vivo* recombinant proteins were also studied, detecting a higher clearance rate for streptokinase (from *S. equisimilis*) and higher immunogenicity of the recombinant BM 86 antigen (from *B. microplus*) when it was used as a vaccine.

S2

Cleavage of the Transferrin Receptor is Influenced by the Side Group at Position 104

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The transmembrane receptor for the iron carrying molecule, transferrin, has a single O-linked carbohydrate at Thr 104, in the external domain near the membrane. This carbohydrate helps to protect the transferrin receptor (TfR) from proteolytic cleavage between Arg 100 and Leu 101, only four amino acids from the site of O-linked carbohydrate attachment. The cleaved, soluble form of the TfR has been found in the medium of cultured cells as well as in human serum and is used as a diagnostic indicator of iron deficiency. We investigated what features at position 104 protect the TfR from cleavage. By site-directed mutagenesis, six different amino acid substitutions were made at position 104. The side chains varied in size and charge. Measurement of the soluble TfR in the cultured medium of cells transfected with each of the mutant TfRs showed that the large and charged side chains inhibited TfR cleavage the most. The mutant TfRs are normal with respect to homodimer disulfide bonding, affinity for transferrin, and proportion of the TfR at the cell surface. In addition, removal of the sialic acid component of the carbohydrate from wild-type TfR by treatment of live cells with neuraminidase enhances TfR cleavage. Expression of wild-type TfR in CHO 1d1D cells (a glycosylation defective cell line) also shows enhanced TfR cleavage under conditions that produce truncated or no O-linked carbohydrate. These results show that the terminal negatively charged sialic acid is important for protection from proteolytic cleavage, and suggest that cleavage could be regulated in the cell by removal of all or part of the carbohydrate.

S2

The Study of Transferrin Biological Action Dependence on Sialic Acid Contents of its Molecules

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The possible dependence of some transferrin (Trf) biological actions (its antioxidant and cell proliferation stimulating effects) on the sialic acid (SA) content of its molecules was studied in isolated rat bone marrow cells. Human Trf was separated into two forms with different content of sialic acid, using ion-exchanged chromatography on DEAE-cellulose. One Trf form (Trf I) contained less SA residues than the other one (Trf II) – on average 3 and 4 per one Trf molecule, respectively. The effects of these molecular Trf forms on Fe^{3+}–ADP stimulated lipid peroxidation (LP), detected by the production of malonic dialdehyde, and replicative DNA synthesis, evaluated by ^{3}H-thymidine incorporation into DNA of isolated bone marrow cells. It was found that both apo-Trf forms inhibited LP, both 2Fe-Trf forms induced ^{3}H-thymidine incorporation into bone marrow DNA. However, apo–Trf II, containing more sialic acid residues than apo–Trf I, inhibited LP in bone marrow cells more efficiently than apo–Trf I. At the same time the effects of apo-Trf I and apo–Trf II on LP in the cell-free system (egg's yolk lipoproteins) were identical. Unlike the action of apo–Trf on LP the effect of 2Fe-Trf on ^{3}H-thymidine incorporation into DNA was more pronounced in the case of low sialylated Trf (Trf I): it stimulated incorporation of tracer twice that of control, while Trf II enhanced it only 1,4 times. The data obtained show that the degree of Trf sialylation significantly influences its biological action manifestation and specifically its antioxidant effects and DNA synthesis stimulation. It may be suggested that the distinctions observed in the biological effects of two Trf molecule forms with different SA content may be explained by unequal bone marrow cell membrane binding of these Trf forms.

S2

Biosynthetic Modulation of Sialic Acid-Dependent Virus-Receptor Interactions

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Sialic acids belong to the essential components of the plasma membrane receptors of many microorganisms including viruses. Synthetic, N-substituted D-mannosamine derivatives can act as precursors for structurally altered sialic acids incorporated into glycoconjugates *in vivo* [1].

In this study we have analysed the potential of these sialic acid precursor analogues to modulate sialic acid-dependent biological functions. The biosynthetic modification of host cell sialic acids by these derivatives drastically and specifically interfered with sialic acid-dependent infection of two distinct primate polyomaviruses. Both, inhibition (over 95%) and enhancement (up to seven-fold) of virus binding and infection were observed depending on the *N*-acyl substitution at the C5 position of sialic acid. These effects were attributed to the synthesis of virus receptors, carrying elongated *N*-acyl groups, with altered binding affinities for virus particles.

Furthermore we could show that the relative amount of biologically synthesized nonphysiological *N*-acyl neuraminic acids directly reflects to the biological effects.

Thus, the principle of biosynthetic modification of sialic acid by application of appropriate sialic acid precursors to tissue culture or *in vivo* offers new means to specifically influence sialic acid-dependent ligand-receptor interactions and could be a potent tool to further clarify the biological functions of sialic, in particular its *N*-acyl side chain.

1. Kayser H, Zeitler R, Kannicht C, Grunow D, Nuck R, Reutter W (1992) *J Biol Chem* **267**: 16934–38.

S2

Structure and Function of a Thrombin-like Snake Venom Enzyme, Batroxobin

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Batroxobin is a thrombin-like enzyme purified from snake venom of *Bothrops atrox, moojeni*. In contrast to thrombin which converts fibrinogen into fibrin by splitting off of fibrinopeptides A and B, batroxobin splits off only fibrinopeptide A. The complete amino acid sequence and carbohydrate structure have been elucidated. The positions of six disulfide linkages have now been determined which differentiated batroxobin from thrombin or trypsin. The role of the carbohydrate moiety in the enzymatic activity of batroxobin has been examined by sequential digestion of the native batroxobin with sialidase, β-*N*-acetylhexosaminidase, α-mannosidase and PNGase F. Sialidase digestion did not influence the enzyme activity significantly, but a remarkable increase in the activity was observed on further treatment with β-*N*-acetylhexosaminidase and α-mannosidase, the latter resulting in M1 batroxobin with 1.9 times greater activity compared to the native batroxobin. The modification of the carbohydrate moiety accelerated the clearance of batroxobin from the circulation. By constructing a molecular model of batroxobin based on comparison with well-characterized thrombin and kallikrein, it was possible to predict the role of carbohydrate in the enzyme activity.

S2

Aprocrine Secreted Proteins of the Rat Coagulating Gland are Glycosylated in an Unconventional Manner

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In the rat coagulating gland we have identified two glycoproteins (secretory transglutaminase and a 29 kDa protein) which are secreted via the apocrine export pathway. Our immunoelectron microscopic studies gave clear evidence that the proteins do not pass through the classical ER/Golgi route during intracellular transport [1]. Consequently, synthesis and glycosylation must take place in the cytoplasmic compartment. In current studies we are analysing the carbohydrate structures using several methods (GC-MS, HPAE-PAD and lectin screening on Western blots). Our data indicate that monosaccharide composition is not compatible with that of conventional N- and O-linked glycans and that the glycan structures are more complex than the O-linked GlcNAc modifications of nuclear pore and cytoplasmic proteins already described [2]. This is in agreement with the fact that it was not possible to release the carbohydrate side chains from the polypeptide by specific endoglycosidases and chemical cleavage methods (β-elimination and HF-treatment). At present we are trying to release the glycans by automated hydrazinolysis. Since glycosylation must take place in cytoplasm, novel structures have to be expected. Further cell biological studies will show the exact site of glycosylation in the cytoplasm. Our morphological findings favour the model that membrane associated glycosyltransferases of the Golgi are competent for carrying out glycosylation processes even at the cytosolic side of the membranes.

1. Steinhoff M, Eicheler W, Holterhus PM, Rausch U, Seitz J, Aumüller G (1994) *J Cell Biol* **65**: 49–59.
2. Hart GW, Haltiwanger RS, Holt GD, Kelly WG (1989) *Annu Rev Biochem* **58**: 841–47.

S2

Functional Role of Intramolecular High-Mannose Chains in the Folding and Assembly of Soybean (*Glycine max*) Lectin Polypeptides

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Soybean lectin, denatured in 6 M guanidine hydrochloride, was quantitatively reconstituted to the active tetrameric structure by simple dilution. The combined use of spectroscopy and size analysis by gel filtration revealed that both the folding and assembly of denatured subunit polypeptides were completely prevented in the presence of 300 μM $Man_9GlcNAc_2Asn$ (M9-Asn), whereas $Glc_{1\text{-}3}Man_9GlcNAc_2Asn$(GM9-Asn) interfered only with the polypeptide assembly. M9-An and GM9-Asn were also able to dissociate the native lectin into subunits, but failed in unfolding them. M5-Asn, which corresponds to M9-Asn devoid of four mannose α1,2 residues, interfered with the reconstitution as well, although less effectively than M9-Asn. Periodate oxidation of the native lectin, resulting in the trimming of the sugar chains to $Man_2GlcNAc_2$, did not impair the refolding of denatured polypeptides, but eliminated the ability of the folded monomers to reassemble to the tetrameric structure. Essentially the same results were obtained with the lectin exhaustively digested with α-mannosidase. Further, completely deglycosylated polypeptides had no ability to refold. These results suggest that the α1,6 arm on the β-mannosyl residue of the high-mannose chains functions in the subunit assembly, whereas other part including the core structure and the α1,3 arm on the β-mannosyl residue contributes to the folding of the subunit polypeptides.

S3. NEW METHODS OF GLYCOCONJUGATE ANALYSIS

Supported by Takara Shuzo Co.

Chairs: Bengt Lindberg, Y. C. Lee

S3. 4.00pm

High Sensitivity MS Strategies for Characterizing Novel Glycans

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By combining micro-chemical and biochemical techniques with mass spectrometry we are successfully characterizing a wide range of novel glycan structures isolated from parasites, pathogens and higher animals. In an important new instrumental development, a laser source has been fitted to our ultra-high sensitivity sector array detector mass spectrometer (ZAB 2SE-FPD) thereby enabling the acquisition of mass spectral data at high resolution and high mass accuracy in the MALDI experiment. We are integrating this laser technology into the strategies we employ based on FAB-MS and LC-ES-MS complemented by protease, glycosidase and oxidative degradations coupled with micro-derivatization.

We have used the above MS methodology to define the structures of the major O-glycans in the cercarial glycocalyx (GCX) of *S. mansoni*. We have discovered that a β-GalNAc(1-4)β-GlcNAc(1-3)α-Gal motif substituted at the 3-position of GlcNAc with an oligofucosyl moiety constitutes the repeating unit in the GCX O-glycans. The surface glycoproteins of *H. contortus* carry N-linked glycans and, like GCX, these are characterized by a high level of fucose. MS analyses of detergent extracts from *H. contortus* suggest that truncated multifucosylated core structures are major constituents of the N-glycans.

In collaboration with Dr M. Virji and colleagues (Oxford) we have established that meningococcal pilin in a glycoprotein. We have examined material released by reductive elimination from several pilin variants and have evidence for the presence of a digalactosyl-containing trisaccharide in all variants other than GalE mutants which lack galactose.

S3. 4.25pm

Glycosylation Site Mapping by MALDI Mass Spectrometry

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We have developed a strategy for glycosylation site mapping based upon matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS). In this strategy, the protein backbone is cleaved enzymatically and a portion of the resulting peptide/glycopeptide mixture is analysed directly by MALDI-MS. A second aliquot of the digested glycoprotein is further treated with PNGase F to release the N-linked carbohydrate chains, followed by MALDI-MS of this sample. The N-linked glycopeptides in this mixture can be easily identified by comparing these two MALDI-MS spectra, since the molecular weights (MWs) of these glycopeptides decrease by the mass of their carbohydrate chains while the peptides and the O-linked glycopeptides are unaffected. A third aliquot of the digested glycoprotein is treated with anhydrous hydrogen fluoride, which removes both the N- and the O-linked chains, without affecting the peptide backbone. The O-linked glycopeptides can then be identified by comparison of this MALDI-MS spectrum to that from the untreated mixture in a manner analogous to that used for identifying the N-linked glycopeptides. These comparisons provide the mass of each carbohydrate chain and the peptide to which it was attached. Combining this information with the amino acid sequence of the protein identifies the glycosylation sites. The primary advantages of our MALDI-MS approach are its simplicity and sensitivity. Unlike the other procedures currently used for glycosylation site mapping, our approach does not require time consuming chromatographic separations. In addition, we have been able to use this procedure to map the glycosylation sites from *femto*mole quantities of glycoproteins.

S3. 4.38pm

^{1}H-NMR Spectroscopy as a Continuous Flow On Line Detection System for Glycolipids Separated by HPLC (LC-NMR)

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Glycolipid separation by HPLC is usually monitored by HPTLC of separated fractions. As glycolipids both differ in, and separate according to, their carbohydrate as well as their ceramide part, pooling of fractions for further separation or structural work is usually difficult without more specific data.

A straight phase silica column HPLC-system connected on-line to a ^{1}H flow-probe of a 500 MHz NMR instrument (Bruker AMX 500), was used. NMR spectra were recorded continuously during 40 min of HPLC running using a gradient system of $CHCl_3$:CD_3OD:D_2O from 80:20:1 by vol, to 60:35:8 by vol. A mixture of lactosyl-, globotriaosyl-, and globotetraosylceramide (300 μg each) was used for testing. On 40 min of continuous recording, 50 spectra (increments) of 32 scans each were recorded. The results could be visualized as single spectra (in total 50 spectra) or as a 3D map. TLC analyses were performed on all collected fractions and the results compared with NMR spectral data at identical elution times. Separate NMR recordings of the total nonseparated glycolipid mixture were performed with different solvent mixtures for identification purposes.

The results show that spectra recorded, although distorted by e.g. solvent effects, contains relevant data and identify glycolipids during chromatography. Signals originating in anomeric protons were easily detected from sample amounts of 30–50 μg calculated to be present in the probe during recording. The difference in, and comparison of, dynamic changes in intensity of different signals during chromatography, may be used to determine molecular origin in only partially separating mixtures.

S3. 4.52pm

A Method for the Analysis of the Linkage Position of Sialic Acid and the Successive Saccharide Using Methylation, Reduction and Trifluoroacetolysis Followed by GC/MS

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A method is under development for the analysis of the ketosidic bond between sialic acid and the first successive saccharide in oligosaccharides and gangliosides. Methylation is performed using methyl iodide in the presence of sodium hydroxide in a dimethyl sulfoxide solution [1]. Subsequent reduction in diethyl ether with lithium aluminium hydride [2] gives a free primary alcohol on the sialic acid C1 position. Trifluoroacetolysis is then performed at 60 °C in 1% trifluoroacetic acid in trifluoroacetic anhydride. The primary alcohol is readily trifluoroacetylated causing a stabilization of the ketosidic bond towards trifluoroacetolysis. Unstabilized glycosidic bonds undergo trifluoroacetolysis prior to trifluoroacetylation, thus yielding a mixture of derivatized monosaccharides and one or more sialo units linked to a saccharide. The mixture is analysed by GC/MS and through evaluation of retention times and mass spectra the different components can be identified.

This method is of importance for the analysis of larger oligosaccharides and gangliosides recovered from different biological sources. The aim is to be able to determine the sialic acid linkage(s) and to identify the successive saccharide in order to evaluate the sialic acid dependent binding of microbes.

1. Larson G, Karlsson H, Hansson GC, Pimlott W (1987) *Carbohydr Res* **161**: 281–90.
2. Karlsson K-A (1974) *Biochemistry* **13**: 3643–47.

S3. 5.05pm

Three-Dimensional Mapping Technique of Pyridylaminated N-Linked Oligosaccharides: Elucidation of Structures of $\alpha5\beta1$ Integrin

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The three-dimensional mapping technique, a new method for structural analysis of N-linked oligosaccharides, has recently been developed. A PA-derivatized oligosaccharide mixture is first separated by HPLC on an anion exchange (DEAE) column and the elution data are placed on the Z-axis. Neutral, and mono, di, tri, and tetra-sialyl oligosaccharides are then individually separated on both an ODS-silica (X-axis) and an amide-silica (Y-axis) column, under the same chromatographic conditions. We then compare the coordinate of the sample with those of known standard PA-oligosaccharides. This mapping technique can differentiate even closely related Neu5Ac$\alpha2 \rightarrow 6$ from $\alpha2 \rightarrow 3$-containing oligosaccharides, and Neu5Ac from Neu5Gc-containing oligosaccharides.

In the integrin receptor, N-glycosylation is essential for association of the $\alpha5$ and $\beta1$ subunits and for the optimal binding to fibronectin. The detailed oligosaccharide structures of $\alpha5\beta1$ integrin receptor were analysed using the 3-D mapping technique. Ten neutral, 6 mono-sialyl, 10 di-sialyl, 7 tri-sialyl, and 2 tetra-sialyl oligosaccharides were isolated from the protein and their structures were characterized. *N*-acetylneuraminic acids were linked predominantly by $\alpha2 \rightarrow 3$ linkage. Fully $\alpha2 \rightarrow 3$ sialylated oligosaccharides constituted 38.1% of the total.

S3. 5.25pm

New Technology for Glycosphingolipid Analysis by Using TLC Blotting

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A new technology for glycosphingolipid (GSL) analysis by TLC blotting has been developed. GSLs on an HPTLC-plate were blotted to a PVDF membrane as follow. The plate was dipped in a mixture of isopropanol: 0.2% $CaCl_2$:MeOH (40:20:7, by vol). Then a PVDF membrane and a glass fibre filter were placed on the plate, and the assemblage was pressed with 180 C iron for 30 s. GSLs blotted on the membrane can be used for the following analyses. (1) GSLs on the membrane can be visualized with chemical reagents such as orcinol, resorcinol reagent as well as immunological staining. (2) GSLs on the membrane can be extracted with MeOH. By this method, 20 GSLs and 12 acidic GSLs separated with 2D-TLC were purified as homogeneous bands. (3) GSLs on the membrane can be analysed directly by MS. More than 15 GSLs obtained from 10^7 cells could be analysed by the MS analysis. (4) GSLs blotted on the membrane could be used as substrates for glycosyltransferase and glycosidase. The reaction products were determined by immunostaining. Further practical applications for GSL analyses of the TLC blotting will be discussed.

S3. 5.40pm

Heterocyclic Derivatives of Reducing Glycans

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Hydrazinolysis is an established technique for the release of both N- and O-linked glycans from glycoproteins. The glycans are initially isolated as the hydrazones, which can be deblocked and recovered in the reducing form. Alternatively, the hydrazones can be converted directly to derivatives which enhance their separation, detection and spectroscopic properties.

Glycan hydrazones exist almost solely in the cyclized form in aqueous solution. As such, they can be considered as glycosyl hydrazines and can be converted into heterocyclic compounds by standard condensation reactions [1]. Accordingly, glycan hydrazones from the hydrazinolysis of fetuin were converted,

without prior purification, to glycosylpyrazoles by condensation with pentan-2,4-dione (acetylacetone). The conversion is rapid at room temperature and the glycosylpyrazoles have excellent properties for reversed- and normal-phase HPLC and CE, as well as good properties for electrospray-mass spectrometry.

Similarly, glycosylhydrazines can be condensed with esters of acetoacetic acid to form glycosylpyrazolones, which can be used in coupling reactions to incorporate functional groups to provide enhanced separation and spectroscopic properties.

1. PCT Patent. 'Glycoconjugate Analysis', No. PCT/AU94/00764.

S3. 5.53pm

Enzymatic Release of Complex Type Oligosaccharides from Native Glycoproteins Using Endo-β-N-Acetylglucosaminidase HS

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The discovery of the endo-β-N-acetylglucosaminidases provided the ability to release intact oligosaccharides from glycoproteins without destruction of the protein moieties. It is desirable to obtain new endo-β-N-acetylglucosaminidases with high specificity for complex type oligosaccharides of intact glycoproteins because of the variety of the structure of Asn-linked oligosaccharides and the limited substrate specificities of known endo-β-N-acetylglucosaminidases.

We found the deglycosidation of human salivary α-amylase family A, which has a biantennary complex type oligosaccharide having fucose linked to the proxymal N-acetylglucosamine, in human saliva [1]. The deglycosidation was catalysed by endo-β-N-acetylglucosaminidase, named endo-β-N-acetylglucosaminidase(endo) HS [2]. Endo HS originated from epithelial cell of human oral cavity epithelium and was an intrinsic plasma membrane protein. It was solubilized and purified in the presence of CHAPS. Endo HS completely acted on human transferrin, calf fetuin and human urinary RNase UL, but, it did not act on ovalbumin, bovine pancreatic RNase B and Taka-amylase. Endo HS also released oligosaccharides from Asn-oligosaccharides prepared from human transferrin, human lactoferrin and calf fetuin, but not Asn-oligosaccharides derived from ovalbumin. Endo HS specifically released bi, tri and tetraantennary complex type oligosaccharides from native glycoproteins even in the presence of core fucose residue.

Thus endo HS can be used for typing the structure of Asn-linked oligosaccharides or investigating the function of complex type oligosaccharides of glycoproteins.

1. (1992) *J Biochem* **112**: 88–92.
2. (1993) *J Biol Chem* **268**: 16074–81.

S3. 6.07pm

Exosequencing: A Novel Approach For Sequencing Heparin/Heparan Sulphate Saccharides

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Structural analysis of glycosaminoglycans (GAGs), in particular the highly complex sequences found in heparan sulphate (HS) and heparin, presents considerable difficulties. Current methods are laborious and generally provide only indirect (and often incomplete) sequence information. We have devised a novel approach by utilizing degradative lysosomal exoenzymes (which remove specific sulphate groups or monosaccharide residues from the non-reducing end of saccharides). Sequential degradation using these exoenzymes in concert with high resolution gradient PAGE mapping techniques provides the basis for development of new strategies for direct sequencing of GAGs. For example, a purified heparin hexasaccharide of structure IdoA(2S)-GlcNSO3(6S)-IdoA(2S)-GlcNSO3(6S)-IdoA(2S)-AMannR(6S) resolves as a single major band on gradient PAGE. Sequential treatment with the enzymes iduronate-2-sulphatase, iduronidase and glucosamine-6-sulphatase (which specifically remove non-reducing terminal 2-O-sulphate groups, iduronate moieties and 6-O-sulphate groups respectively) resulted in downward band shifts due to lower molecular weight of the products. The banding pattern can be interpreted to read directly the sequence of the non-reducing end of the hexasaccharide. The results demonstrate the utility of this novel approach for sequencing GAG saccharides.

S3 POSTERS

S3

Structural Characterization of a Glycosphingolipid Mixture from Pig Lungs with LC-NMR and MS-MS

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Pig is a possible donor in human xenotransplantation. The lung is rich in endothelial cells known in aorta to express Galα1-3neolactotetraocylceramide – one major target for human anti-pig antibodies. Glycosphingolipids from four pig lungs were prepared according to the method of K-A Karlsson, with slight modifications.

The glycosphingolipids were purified and separated according to sugar chain length on an LKB HPLC. Two native glycosphingolipid fractions with (1) 1–5 sugar residues, and (2) ≥ 4 sugar residues were used for structural characterization. A Bruker LC 22C pump with a straight phase silica column was connected on line to a Bruker AMX 500 MHz spectrometer equipped with a dedicated ^1H flow probe with a cell volume of 160 μl. Continuous flow ^1H spectra were obtained using a one dimensional NOESY pulse sequence including presaturation. An acquisition time of 1 s was used. The total time was 40 min with 50 increments of 32 scans each. Fractions of 3.5 mg were eluted in 100 μl of the solvent (i.e. 300 μg per component). The gradient systems used were mixtures of $CHCl_3$:CD_3OD:D_2O.

The MS-MS experiments were carried out on Fison Autospec OA TOF equipment. The glycolipid samples were used in both a derivatized (i.e. methylated) and native form in the experiments. Our data from LC-NMR and MS-MS analysis indicate a future potential for the combined methods in the structural characterization of glycosphingolipid based antigen mixtures of biological origin.

S3

Acoustic Cavitation and Glycosylation

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The acoustic cavitation on a multicomponent aqueous system (Adenine, ribose, CF_3COONa, $ClCH_2CONH_2$) has been studied under different conditions of acoustic power and composition and the following results have been obtained:

1) Low acoustic power (≈ 1 W cm^{-2}): the system was sonicated at 20 °C. After 90 h of sonication the liquid phase changed to a lilac-pink colour and successively the solid phase also changed. A change of colour was also observed in a non-sonicated mixture, but over a longer period. The pink powder was fluorescent: its molecular spectra were similar to adenine, but there were differences in reactivity.

2) Medium acoustic power (90 W cm^{-2}). The conjugation of nitrogen present in the imidazole ring of adenine with the CH_2CONH_2 group was easily obtained at 20 °C. When the sonicated mixture was warmed to ≈ 70 °C a moderate amount of adenosine was obtained, i.e. the glycosylation was obtained.

3) High acoustic power (≈ 500 W cm^{-2}). This power was principally a destroying agent and glycosylation was not obtained. Modifications of adenine were observed depending on the presence in the reacting medium of other substances. For example the role of CF_3COONa was tested. Since it absorbs O_2 strongly (as we have demonstrated recently), this probably reduced the presence of oxygen in the cavitation bubble, and consequently the temperature in both the collapsing bubble and the liquid around the bubble. The liquid around the bubble is the site where the reactions of adenine occur, owing to its very low vapour pressure.

S3

Density Gradient Electrophoresis Revealed Distinct Populations of Human High-M_r mucins (MG1) Secreted by Different Salivary Glands

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High-M_r mucins (MG1s), heavily glycosylated with O-linked oligosaccharides, are major constituents of human saliva. MG1s originating from individual salivary glands were chemically characterized. The carbohydrate content of MG1 derived from palatal (PAL), submandibular (SM) and sublingual (SL) saliva was typical for mucins but showed heterogeneity, especially in the amount of sialic acid and sulfated sugar residues. The physicochemical properties of native MG1s make conventional SDS-PAGE and ion-exchange chromatography unsuitable for investigating differences between individual MG1 mucins. Recently, a Density Gradient Electrophoresis (DGE) device has been developed, primarily for separation based on the charge of entire cells or cell organelles [Tulp *et al.* (1994) *Nature* **369**: 120–26]. We have used this device to study the various high-M_r salivary mucins. MG1s of the individual glands clearly expressed distinct electrophoretic mobilities, as monitored by ELISA using MG1-specific mAbs. Even within a particular MG1 preparation subpopulations could be distinguished. DGE analysis of chemically and enzymatically modified MG1 series, followed by ELISA and dot-blot detection using specific mAbs, lectins, and high-iron diamine (HID) staining, suggests that the high electrophoretic mobility of PAL-MG1 stems mainly from a high sulfate content, whereas the distinct SL subpopulations differ mainly in binding-type and the amount of the sialic acid residues. SM-MG1 mostly resembles the low mobile subpopulation of SL-MG1 possessing, however, a lower sulfate content. In conclusion, DGE appears to be a powerful method for native mucin analysis, leading to the direct result that MG1s from the various salivary glands are biochemically much more diverse than was previously assumed.

S3

Unmodified Glycosaminoglycan Oligosaccharides for Structure-Function Studies

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Glycosaminoglycan (GAG) polysaccharides of cell surface proteoglycans interact with a range of proteins involved in cellular adhesion, motility and proliferation. One approach to investigating molecular interactions of this type entails the use of defined oligosaccharide fragments in activation and inhibition assays. Although GAG oligosaccharides from enzymatic or chemical depolymerization are employed in such investigations the modified terminal residues of fragments may interfere with activities.

In the present report, an oxymercuration procedure [1] to cleave unsaturated uronic acid (ΔUA) residues from glycan lyase-produced fragments is assessed by characterizing both the cleaved ΔUA and the residual saccharide structures. This is shown initially with heparin disaccharides by conversion of the products to neoglycolipid derivatives and analysis by TLC-LSIMS. Multiple but characteristic products are detected that permit identification of terminal ΔUA and its sulfate content, and these are used in structure elucidation of several heparin fragments, including tetra- and hexasaccharides, where variation in ^{1}H-NMR chemical shifts make ΔUA assignment uncertain. Glycosidic linkages and O- and N-sulfate groups are shown to be preserved in the residual structures after removal of ΔUA from heparin di- and tetrasaccharides, and chondroitin tetra- and hexasaccharides. Our results verify the use of the oxymercuration reaction for generating series of unmodified GAG fragments from proteoglycan and other sources for biological activity studies, while TLC-LSIMS of neoglycolipid derivatives provides a means for sensitive determination of the composition and sequence of short GAG chains.

1. Ludwigs *et al.* (1987) *Biochem J* **245**: 795–804.

S3

Analysis of 9-Aminopyrene-1,4,6-trisulfonate Derivatized Sugars by Capillary Electrophoresis with Laser-induced Fluorescence Detection

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Reductive amination of reducing sugars with 9-aminopyrene-1,4,6-trisulfonate (APTS) results in the formation of adduct that is substantially more intense yellowish green colour than that of

the APTS. Fructose and other ketohexoses can be derivatized by a similar procedure. The APTS-mono and oligosaccharides were characterized by capillary electrophoresis with laser induced fluorescence (CE/LIF). APTS derivatized sugars have λ_{max} at 455 nm with a significant absorption at 488 nm (35% λ_{max}) while the APTS has a λ_{max} at 424 nm with little absorption at 488 nm. APTS derivatized sugars fluoresces at 512 nm, while APTS fluoresces at 501 nm. Selective fluorescence detection of the APTS-sugar adducts was achieved by using excitation at 488 nm of argon-ion laser and fluorescence emission at 520 nm. The detection limit of the CE/LIF system for purified derivatized sugar is 0.4 nM (S/N = 10). The lowest amount of oligosaccharide which can be derivatized and detected by the present method is 1.0 pmol.

APTS-mono- and oligosaccharides are well-separated by CE/LIF in many different buffer systems without relying on borate complexation. APTS-oligosaccharide ladders are well-resolved up to 50-mers in phosphate buffer at pH 2.2 by CE/LIF system. APTS-maltohepatose and sialylactose were used to probe the specificity of amylases and neuraminidase, respectively. Analysis of glycoprotein-associated oligosaccharides using standard cleavage procedures along with APTS-derivatization and the CE/LIF method will be discussed. The higher detection sensitivity, derivatization efficiency and separation power of the APTS derivatization in the CE/LIF analysis procedure provide substantial improvement over the existing method of carbohydrate analysis.

S3

The Use of Capillary Electrophoresis in the Analysis of N-Linked Oligosaccharides from Glycoproteins

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The demand for easy to perform methods for carbohydrate analysis is rapidly increasing. The use of capillary electrophoresis in glycoprotein analysis offers the advantages of high resolution, small sample size, reproducible micro-quantitation, as well as attractive opportunities for automation of the analytical process. Capillary electrophoresis (CE) was used to estimate degree of glycosylation through comparative peptide mapping protocols, using native and de-glycosylated peptide digests of a glycoprotein. A CE separation system and a sample preparation protocol were optimized for the analysis of enzymatically released N-linked oligosaccharides, labelled with 1-phenyl-3-methyl-5-pyrazolone (PMP). High-resolution oligosaccharide 'fingerprints' were obtained. Exoglycosidase treatment, followed by CE analysis, allowed identification of the oligosaccharide terminal sugars, and their regiospecific linkages. Further characterization of the released oligosaccharides, with tentative structural assignment, was possible on the basis of data obtained from composition analysis and enzymatic treatment, in combination with information available from the literature. Composition analysis of purified oligosaccharides was carried out using one hydrolysis condition for the release of neutral and amino sugars, and another for sialic acids. Upon PMP-labelling, and in the case of sialic acids after enzymatic conversion to mannosamine derivatives followed by PMP labelling, complete composition analysis of the released monosaccharides was achieved with high selectivity and efficiency, using micellar electrokinetic chromatography (MEKC).

S3

N-linked Glycan Profiling of Native and Recombinant Glycoproteins Using a Modification of the Fluorophore-Assisted Carbohydrate Electrophoresis Methodology

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Several methods have recently been developed for oligosaccharide profiling in natural and recombinant glycoproteins to characterize both N and O linked oligosaccharides. Among these, FACE (Fluorophore-Assisted Carbohydrate Electrophoresis) is one of the simplest and least time consuming but there are still some difficulties which could be solved.

We have introduced some modifications to the classical FACE for N-linked glycan profiling which include the addition of triethylamine on the running buffer giving rise to a 'real' size of charged oligosaccharides having different sialic acid units. The approach to a classical HPLC analysis using NH_2 column of the 8 amine naphthalene trisulfonic acid derivatives (oligosaccharide-ANTS derivatives) is also discussed, showing a well-resolved set of peaks that are useful and easily recovered for mass spectrometry.

Some examples will be given which include native secreted glycoproteins from fungi (cellobiohydrolases and endoglucanases, cellulases) and also recombinant proteins expressed in yeast (endo α1-6 glucanhydrolase, dextranase, and β-fructofuranoside fructohydrolase, sucrose invertase) and in mammalian cells (erythropoeitin in CHO cells).

S3

Detection of Gastric Mucin on PVDF Membrane Using Histochemical Staining Methods

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Mucin, a high molecular weight mucus glycoprotein, is a major component of the gastric mucus which is considered to protect the mucosa from gastric acid, pepsin, etc. Gastric mucin has been histochemically characterized. The results showed that there are many kinds of mucin which differ from each other based on their carbohydrate chains and that the location of each mucin is different in the tissue. To detect and identify the individual mucin, the extracted mucins, after fractionation by various gel chromatography and density gradient centrifugation methods, were blotted on a PVDF membrane. The membranes were then stained using periodate-Schiff (PAS), Alcian blue (AB), high-iron diamine (HID), galactose oxidase-cold thionin Schiff (GOCTS) or paradoxical concanavalin A staining (PCS) methods. The results showed that gastric mucins on the membrane were quantitatively detected by these assays and that gastric mucins were eluted at the void volume position during Sepharose CL-4B chromatography. Mucins were also detected during CsTFA density gradient centrifugation. Mucins detected by PAS were excluded from a Sepharose CL-2B column, but most of them were included in the same column after reduced alkylation. In contrast, mucins detected by PCS were excluded from the same column even after reduced alkylation. The individual mucins were also detected during Q-Sepharose chromatography according to their negative charges. Thus, this

blotting method is useful for detecting individual mucins during gel chromatography and density gradient centrifugation.

S3

Structural Determination of N-Linked Oligosaccharides by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

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N-Linked oligosaccharides were released from glycoproteins by hydrazinolysis and by PNGase-F digestion and examined by MALDI using 2,5-dihydroxybenzoic acid as the matrix. Strong MNa^+ ions were obtained from the underivatized compounds with detection limits in the low pmol range. Profiles were obtained from the N-linked oligosaccharides released from several glycoproteins including ribonuclease, ovalbumin, human IgG and the parotid gland. Quantitative studies with standard oligosaccharides showed good correlation between sample amount and recorded signal with little variation with oligosaccharide structure. Increased sensitivity (mid fmol region) was achieved with reducing terminal derivatization to incorporate a cationic site. The technique was used to monitor the products of exoglycosidase digestions used for structural determination studies. Greatly improved resolutions were obtained on a magnetic sector mass spectrometer (VG AutoSpec) compared with that obtainable on a linear time-of-flight (TOF) instrument. Fragmentation, providing information on sequence and branching was present in the spectra recorded on the magnetic sector instrument. Fragment ions were mainly the products of glycosidic cleavage with additional ions produced by cleavages of the reducing-terminal GlcNAc. Additional experiments using a TOF mass spectrometer fitted with a reflectron and a magnetic sector instrument fitted with an orthogonal-TOF analyser yielded improved fragmentation giving some linkage information.

S3

The Hypothetical N-Glycan Charge: A Number That Characterizes Protein Glycosylation

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The production of recombinant glycoprotein therapeutics requires characterization of glycosylation with respect to sialylation and lot-to-lot consistency. Here we introduce the 'hypothetical N-glycan charge' ('Z') as a parameter that allows the characterization of the protein glycosylation in a simple and efficient manner.

The hypothetical N-glycan charge Z of a given glycoprotein is deduced from the N-glycan mapping profile obtained via HPAE-PAD. In HPAEC, N-glycans are clearly separated according to their charge, i.e. their number of sialic acid residues, exhibiting retention times in the range of 13–18 min (asialo, as), 20–23 min (monosialo, MS), 27–32 min (DiS), 34–38 min (TriS), and 40–43 min (TetraS structures), using the validated gradient 'S' for sialylated N-glycans [1].

Z is defined as the sum of the products of the respective areas under the curve (AUC), each multiplied by the corresponding charge:

$$Z = AUC_{(as)} * 0 + AUC_{(MS)} * 1 + AUC_{(DiS)} * 2 + AUC_{(TriS)} * 3 + AUC_{(TetraS)} * 4$$

Thus, a glycoprotein with mostly C4-4* structures will provide $Z \cong 400$ (e.g. rhu EPO (CHO), Z = 361, or rhu EPO (BHK), Z = 325), a glycoprotein carrying largely C3-3* structures will amount to $Z \cong 300$ (e.g. bovine fetuin, Z = 290), a glycoprotein with C2-2* structures will have $Z \cong 200$ (e.g. human serum transferrin, Z = 207, or human antithrombin III, Z = 180), and a glycoprotein carrying only high-mannose type or truncated structures will provide $Z \cong 0$ (e.g. bovine pancreas ribonuclease B, Z = 15, or hen ovomucoid, Z = 15, respectively) (for nomenclature and abbreviations see [2]).

The determination of Z was validated in multiple repetitive experiments and proved to be highly accurate and reliable. Z may therefore be regarded as a new and characteristic parameter for protein N-glycosylation.

1. Hermentin *et al.* (1992) *Anal Biochem* **203**: 281–89.
2. Hermentin *et al.* (1994) *Anal Biochem* **221**: 29–41.

S3

Characterization of Recombinant Human TSH Glycosylation by Fluorophore Assisted Carbohydrate Electrophoresis and Glycosidase Sequencing

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In humans, thyroid stimulating hormone (TSH) is produced in the pituitary where it is glycosylated with biantennary N-glycans that are sulfated and/or sialylated [1–3]. We have characterized the glycosylation of recombinant human TSH produced in CHO cells using fluorophore assisted carbohydrate electrophoresis (FACE) in conjunction with glycosidase sequencing.

When TSH oligosaccharides are labelled with the fluorophore 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and separated by polyacrylamide gel electrophoresis (FACE) five major bands are visible. When these bands were isolated from gels and digested with sialidase to remove sialic acid residues, four of the five bands resolve into more than one band (as many as three different neutral structures were resolved from one sialylated band). The result is a total of at least 10 different structures in the five original bands. The desialylated bands were isolated from PAGE gels and sequenced further. We have shown that 55% of these desialylated structures were biantennary, 31% triantennary and 14% tetraantennary, with different levels of fucosylation. This heterogeneity in size and charge results in multiple oligosaccharides with similar mass:charge ratios. Because FACE separates oligosaccharides primarily according to their charge:mass ratio, some structures coelute. This makes it necessary to desialylate (eliminating the heterogeneity due to charge) and isolate the neutral structures before doing further sequencing. However, even with this type of heterogeneity FACE is a rapid and sensitive technique for glycosidase sequencing.

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S3

Improved O-Glycosylation Site Mapping of Glycoproteins

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Current methods for determining the sites of O-glycosylation in a given protein call for specific enzymatic (e.g. tryptic) cleavage of the peptide backbone followed by isolating and sequencing the glycopeptide fragments. Amino acid sequencing is typically performed by automated Edman degradation (starting from the N-terminus) or by tandem mass spectrometry. A glycosylated amino acid either fails to show up or appears with a significantly increased molecular mass, respectively. However, both sequencing methods become practically cumbersome for rather large peptides, especially if the latter contain more than one occupied O-glycosylation site. To overcome these problems, we propose to further cleave the isolated glycopeptide(s) non-specifically (e.g. by pronase), and to monitor the appearance of fragment peptides and glycopeptides as a function of the incubation time. Monitoring is conveniently accomplished by MALDI-TOF mass spectrometry. Based on the obtained m/z values for newly generated glycopeptides, the masses of the corresponding peptide backbones can be calculated. If pursued until the peptide backbone is but a couple of amino acids long, the exact site of O-glycosylation in the protein can be determined. The proposed procedure is rapid and sensitive; answers are obtained over the course of a few hours on ~100 pmol of glycopeptide material. We will illustrate the application of this method for the precise O-glycosylation site mapping of bovine fetuin, a glycoprotein with three to four occupied O-glycosylation sites within a single tryptic fragment (^{228}Val-Arg$_{288}$).

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S3

Application of FMOC-Cl for the Quantitative Determination of 1-Amino-Oligosaccharides in the Low Range

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A fluorescence tagging agent, 9-fluorenylmethyl chloroformate (FMOC-Cl) was used for the determination of 1-amino-oligosaccharide intermediates generated from glycoproteins (ex. RNase B) by N-Glycanase (PNGaseF; EC 3.5.1.52). RNase B with N-Glycanase (0.05 mU per μg of protein) resulted in the complete removal of Asn-linked oligosaccharide chains. FMOC-labelled oligosaccharides were separated on an Amide 80 column by HPLC, and monitored at 278 nm for E_x and at 333 nm for E_m by a fluorometric detector. The complete derivatization of FMOC-Cl to 1-amino-oligosaccharide was achieved at 20-fold molar excess of reagent for 60 min at room temperature. More than 99% of 1-amino-oligosaccharides was derivatized with FMOC-Cl. The yield compared with the extent of derivatization of reducing sugars with 2-aminopyridine over 8 h at 90 °C of 71% as reported by Hase *et al*. (1984). When RNaseB FMOC-labelled oligosaccharides were analysed, five peaks corresponding to Man5-9GlcNAc2 were separated by HPLC. Each calibration curve of FMOC-labelled Man5-9GlcNAc2 derivatives was plotted, and all their responses were linear over the range 0.05–1.5 pmol.

S3

The Fluorometric Determination of Oligosaccharides by Capillary Electrophoresis Equipped with a New Detector Cell

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A new fluorescent detector cell was prepared for use in the analysis of capillary electrophoresis (CE) and micro HPLC, and positioned over the conventional capillary tube of both apparatus. The detector cell allowed for the use of less expensive conventional light sources such as a xenon lamp with excellent resolution of the components of a sample being analysed. Reductively pyridylaminated derivatives of glucose oligomers and high mannose type oligosaccharides were used as the components of a sample. The detector was linear over three orders of magnitude and showed detected limits of 0.04–0.16 pmol for oligosaccharides. Although most fluorescence-labelled derivatives have been only detected at a definite wavelength using a light source such as a laser beam, all kinds of fluorescence compounds could be determined by the cell utilized in this study using an inexpensive xenon lamp, instead of a laser beam.

S3

Utilizing Gas Chromatography-Mass Spectrometry and Tandem Mass Spectrometry for Analysing Mixtures of Neutral, Sialylated and Sulfated Mucin Oligosaccharides

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Mucin oligosaccharides released by alkaline borohydride from porcine intestinal glycopeptides were fractionated on an anion exchange column (Karlsson *et al*. (1995) *Glycoconjugate J* **12**: in press). Neutral species were eluted by methanol. A mixture of dimethyl sulfoxide and iodomethane converted the sialylated species into methyl esters. These were then eluted by methanol. Finally the sulfated species were eluted by 1 M pyridinium acetate. The methyl esters of the sialic acid residues could be further derivatized to methyl amides. Characterization of the neutral and the derivatized sialylated species were done by high temperature GC/MS of their permethylated species. The mixture neutral oligosaccharides was separated into 21 different components with the largest one a permethylated hexasaccharide. The sialylated mixture was separated into 28 components involving both monosialylated and bisialylated species with the largest one a monosialylated pentasaccharide. The sulfated species were analysed as native or peracetylated oligosaccharides by collision induced dissociation of their $[M-H]^-$-ions using a four sector tandem mass spectrometer. Fourteen monosulfated structures were deduced from the spectra with the main sulfation site at the C-6 of an *N*-acetylhexosamine attached to the C-6 of the *N*-acetylgalactosaminitol residue.

The same approach applied to an intestinal glycopeptide

fraction from rats infected with the parasite *Nippostrongylus brasiliensis* revealed glycosylation alterations of the sialylated oligosaccharides.

S3

Characterization of the N-Glycans on Recombinant Human Factor VII (rFVIIa) by Liquid Chromatography, Mass Spectrometry and Glycosidase Digestions

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The N-linked glycosylation of pharmaceutical glycoproteins can impact properties of the glycoprotein, e.g. the pharmacokinetics. In order to characterize the N-glycans of rFVIIa produced in baby hamster kidney (BHK) cells, we have investigated the use of liquid chromatography and electrospray mass spectrometry of N-glycans before and after glycosidase digestions according to the reagent-array analysis method (Edge *et al.* (1992) *Proc Natl Acad Sci USA* **89**: 6338–42).

The N-glycans have been released from rFVIIa by hydrazinolysis and purified by ion-exchange chromatography and size exclusion chromatography. The quantitatively dominating glycan structures have by the combination of liquid chromatography and mass spectrometry been characterized as di- and monosialylated corefucosylated biantennary structures. Among the neutral and the monosialylated glycans, structures with galactose substituted with *N*-acetylgalactosamine have been determined. Through the use of a peptide mapping procedure, the N-glycan structures are assigned to the two N-glycosylated sites of rFVIIa. Suitable analyses for documentation of batch-to-batch consistency are proposed based on the results of the structural characterization.

S3

Simultaneous Determination of Glycosaminoglycans in Skin Dermis

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Generally, most dermatopathia like allergic dermatitis are accompanied with a constructive alteration of the skin dermal extracellular matrix (ECM). The alteration is closely correlated with qualitative and quantitative changes of glycosaminoglycans (GAGs). Hence, an analytical method of GAGs, which is applicable to a small piece of sample such as experimental animal's skin or human skin collected for biopsy, would be useful. Major GAGs constructing skin dermal ECM are dermatan sulfate (DS), chondroitin sulfate (CS) and hyaluronan (HA). They consist of characteristic disaccharide units, uronic acid-hexosamine. We therefore, investigated a chromatographic determination method of characterizing disaccharides obtained from these GAGs by solvolysis; DS, CS and HA were quantitatively degraded to *N*-acetyldermosine, *N*-acetylchondrosine and *N*-acetylhyalobiuronic acid in DMSO containing pyridine, and these disaccharides were then determined simultaneously by ion-exchange HPLC equipped with a fluorometric detector using 2-cyanoacetoamide as a post column reagent. We simultaneously and sensitively determined the DS, CS and HA without interferences of other GAGs such as heparan sulfate.

S3

Use of a Nano-NMR Probe for the Analysis of Complex Carbohydrates Available in Microgram Amounts

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Application of NMR spectroscopy to the structural analysis of complex carbohydrates of biological significance is usually limited due to the lack of sensitivity of the method. We explored the use of a new Nano-NMR probe that allows to obtain high-resolution spectra on very small ($< 40\ \mu l$) samples avoiding solvent contaminants and background. The approach was used for the identification of a novel glycosaminoglycan core-like molecule.

It is known that β-xylosides compete with endogenous proteoglycan core proteins acting as alternate acceptors for synthesizing glycosaminoglycan chains. When human melanoma cells were incubated with 1 mM 4-methyl-7-β-xylosyl-coumarin (Xylβ-4MU) and [6-^{3}H]-Gal, one of the isolated products (xyloside I) showed unexpected properties. Since it was resistant to the commonly used glycosidases, we isolated and purified enough material for physico-chemical analysis. Compositional analysis by HPAEC-PAD showed an approximate 1:1:2:1:1 molar ratio of GalNAc, GlcA, Gal, Xyl, and 4-MU. LSIMS and ESIMS indicated that the main component of this mixture of products was consistent with the following structure: $HexNAc_1HexA_1Hex_2Pent_1MU_1$. About 20 μg of this product (in 40 μl of D_2O) were analysed by 500 MHz NMR spectroscopy using the Nano-NMR probe. Using a combination of 1-D and 2-D COSY, TOCSY, and ROESY experiments we identified the main component in this sample as:

GalNAcα(1-4)GlcAβ(1-3)Galβ(1-3)Galβ(1-4)-Xylβ(1-7)-4-MU

The novel aspect of this molecule is the presence of a terminal αGalNAc residue at a position that is normally occupied by β-GalNAc in chondroitin sulfate or by αGalNAc in heparan sulfate chains. The αGalNAc residue at this position could influence the further extension of the glycosaminoglycan chain.

S3

Identification of Glycosaminoglycans Using Fluorophore-Assisted-Carbohydrate-Electrophoresis, FACE®

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A simple, sensitive, monosaccharide analysis to differentiate types of glycosaminoglycans using FACE has been developed. Chondroitin sulfates A and C, dermatan sulfate, heparin and heparan sulfate, and keratan sulfate were hydrolysed using amino sugar hydrolysis conditions (4 N HCl), re-*N*-acetylated, labelled with the fluorophore, 2-aminoacridone (AMAC), and separated by PAGE. After 30 min hydrolysis, the dermatan and keratan sulfates were readily recognizable by the presence of labelled bands from GalNAc and iduronic acid, and GlcNAc and galactose, respectively; whereas, both chondroitin sulfates and heparin/heparan sulfate samples were only partially hydrolysed and gave a different band which we propose to be the

desulfated disaccharides. After 3 h hydrolysis, the chondroitin sulfates and heparin/heparan sulfates were distinguishable by the presence of bands from GalNAc or GlcNAc, respectively. The GlcA lactone band was present predominantly instead of GlcA and the iduronic acid band was not detected (other unidentified weak bands appeared). This method has been used to differentiate the types of glycosaminoglycans from urine and serum. Once the type of glycosaminoglycan has been established, the position of sulfates can be determined chemically by nitrous acid depolymerization or by reaction with specific enzymes. For example, heparin was digested with heparinase to yield disaccharides, and tetrasaccharides which were fluorophore labelled with disodium 8-amino-1,3,6-naphthalene trisulfonate (ANTS) and separated by PAGE. The positions of the IS-IVS disaccharide standard bands from heparin have been determined.

S3

Ceramide Glycanase Assay by Fluorescence Quenching and Energy Transfer Using A Bi-Fluorescence-Labelled Lactoside

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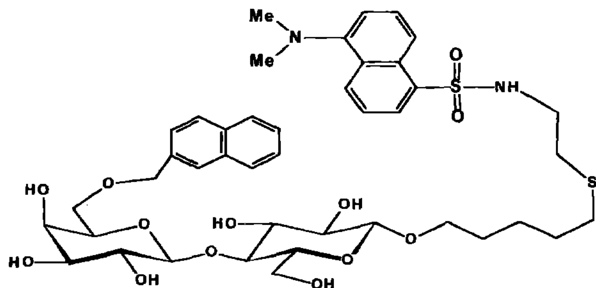
A bi-fluorescence-labelled lactoside as a substrate for ceramide glycanase has been synthesized. Its structure is shown below [1]. This compound has a fluorogenic donor and an acceptor at an N-terminal. The key steps in the synthesis of the substrate were reductive opening of the cyclic acetal [2] and the Michael addition to a double bond.

We found ceramide glycanase (CGase) from the leech *Macrobdella decora* [3] can cleave this substrate. Upon the enzymatic cleave of the linkage between the lactoside and the aglycon, an increase in the donor emission and a decrease in the acceptor emission were observed. These change can be used for sensitive assay of CGase.



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S3

Sequencing and Structural Analysis of Carbohydrates Using Recombinant Glycosidases

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The combination of Fluorophore Assisted Carbohydrate Electrophoresis (FACE) and highly specific cloned glycosidases is a powerful tool for the sequencing of carbohydrates. We have developed a considerable number of these enzymes and have used them in a strategy to sequence oligosaccharides. In many cases, we can determine not only the sequence, but the linkage of the monomers since the enzymes exhibit linkage specificity. With a sufficient number of enzymes, complete enzymatic sequence and linkage analysis should be possible.

S3

Fluorescent Labelling, Separation and Sequencing of N-Glycans

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The structural analysis of subnanomolar amounts of N-glycans derived from natural and recombinant glycoproteins is of increasing interest, as frequently only a limited amount of material from biological sources is available. In the present study we have investigated the suitability of three new commercially available tools, comprising fluorescent labelling of oligosaccharides with 2-aminobenzamide, HPLC-separation and glycan sequencing using an array of specific exoglycosidases (Reagent Array Analysis Method-RAAM) in conjunction with a RAAM 2000 GlycoSequencer Workstation.

Two different approaches have been performed using α_1-acid glycoprotein as a well characterized glycoprotein. In the first approach a total glycan pool obtained by automated hydrazinolysis was fluorescently labelled by reductive amination with 2-aminobenzamide (2-AB). The oligosaccharides were separated in volatile buffer by anion exchange HPLC on GlycoSep C according to their degree of sialylation, subsequently converted to their neutral counterparts by sialidase digestion and then separated on APS-Hypersil. The separated N-glycans were characterized by RAAM sequencing on gel permeation chromatography (GPC) with automated data analysis and with mass determination by MALDI-TOF mass spectrometry.

In a second approach, prior to fluorescent labelling, sialylated and desialylated native N-glycans were first separated by anion exchange chromatography on Mono Q and CarboPak PA-100 respectively and were detected as reducing oligosaccharides by pulsed amperometric detection (PAD) at pH 13. The separated N-glycans were each fluorescently labelled with 2-AB, fractionated by GPC and characterized as described above. The latter approach required desalting and fluorescent labelling of each of the separated sugars and sometimes caused a partial destruction of glycans but offered the possibility of synthesis of neoglycoconjugates from separated glycans in addition to structure analysis.

In summary, the procedure of fluorescent labelling of N-glycans with 2-AB, combined with HPLC-separation, RAAM sequence analysis and MALDI-TOF-MS is a powerful technique which allows a fast and sensitive characterization of subnanomol amounts (<100 pmol) of glycans.

S3

ESIMS and FABMS of Sialylated Lewis-type Glycoconjugates

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The Lewis-type glycoconjugates with acidic functional group(s) at the non-reducing end are of particular interest because they are probably ligands to selectines. Taking advantage of mass spectrometry, which requires much smaller sample amounts to perform detailed structural studies of large biomolecules, we have established methods to distinguish Le^x-type glycoconjugates from the Le^a-type isomers.

In contrast to much more acidic sulfated analogues, both positive and negative ion FAB mass spectra of sialylated Le-type glycolipids in normal as well as in the CID-MS/MS scanning mode indicate that charges are mostly retained on the ceramide side. Thus formed Y-ions clearly show the sugar chain sequences. On the other hand, (–)ESI CID-MS/MS spectra of $(M\text{-}Na)^-$ and $(M\text{-}Na\text{-}H)^{2-}$ of sialylated Le-type glycolipids-Na salts show a line of information complementary to Y-ions including the non-reducing end structures by B-ions, and B- and C-ions, respectively.

These results have proved that ESIMS and FABMS are very useful and reliable methods for structural studies of acidic glycoconjugates, which are found in trace amounts of biological specimens.

S3

N-Glycosylation Site Mapping of Human Plasma α_1-Antichymotrypsin

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We report the structural characterization of the carbohydrate moiety of α_1-antichymotrypsin (α_1-ACT), a serine-proteinase inhibitor (serpin) of major importance in inflammatory processes. The glycoprotein as isolated from human plasma has a molecular weight of about 57.5 kDa, determined by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. The amino acid sequence of the protein (accounting for a molecular weight of 46410 Da) contains six potential N-glycosylation sites (N-X-S/T). To ascertain the presence of carbohydrate at each of these sites, α_1-ACT was first cleaved by CNBr. The resulting (glyco)peptides were separated by reversed-phase HPLC and analysed by MALDI-TOF mass spectrometry before and after N-glycanase digestion. Three glycopeptides were identified, two of which had one occupied N-glycosylation site each. The third glycopeptide which contained four N-X-S/T sequences was further cleaved by tryptic digestion. Only three glycopeptides were identified in the tryptic digest by MALDI-TOF mass spectrometry before and after N-glycanase digestion, complemented by N-terminal amino acid sequencing. The remaining N-X-S/T site in the CNBr-produced multiply glycosylated peptide was retrieved exclusively as a peptide, and is thus not utilized for N-glycosylation in α_1-ACT. The structures of the oligosaccharides on the five individual CNBr(±trypsin)-generated glycopeptides were characterized, where possible, by ^{1}H-NMR spectroscopy, carbohydrate composition analysis (both before and after N-glycanase treatment), HPAE chromatography and electrospray mass spectrometry (after release of the peptide).

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S3

Separation of Neutral Asparagine-Linked (N-Linked) Oligosaccharides by High pH Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC/PAD)

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Since the publication of a paper by Hardy and Townsend [1] describing the separation of N-linked oligosaccharides by HPAEC/PAD, there have been many papers which report the use of HPAEC to separate both charged and neutral mammalian oligosaccharides. It has been reported that HPAEC is poor at separating mixtures of high-mannose and neutral complex oligosaccharides [2]. In this communication we show that an increase in eluent pH improves the HPAEC separation of neutral N-linked oligosaccharides. Using 250 mM NaOH and a gradient of sodium acetate we resolve a mixture of twelve neutral and complex N-linked oligosaccharides in less than 30 min. The separation of these oligosaccharides is generally in the order of increasing size. Two oligosaccharides with hydrodynamic volumes differing by only 0.1 glucose units are baseline resolved. We show that this separation, requiring no oligosaccharide derivatization, is possible on either the CarboPac PA1 or the CarboPac PA100 column.

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S3

Molecular Cloning of α-1,3/4-Fucosidase and an Enzyme Specific for Type 1 Chain

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Two exo-glycosidases, α-fucosidase and lacto-*N*-biosidase are produced by *Streptomyces* sp. strain 142. α-Fucosidase specifically hydrolyses Fucα1-3GlcNAc and Fucα1-4GlcNAc linkages in $Lewis^x$ and $Lewis^a$ structures, but it cannot hydrolyse α-2,3-sialyl $Lewis^x$ or α-2,3-sialyl $Lewis^a$ structures [1]. An enzyme specific for type 1 chains, lacto-*N*-biosidase, acts from the nonreducing termini and produces lacto-*N*-biose (Galβ1-3GlcNAc; 2). The substrate structure essential for enzyme activity is the terminal lacto-*N*-biosyl residue without any substitutions. These glycosidases are useful for structural and

functional analysis of the oligosaccharides in glycoconjugates. To identify the primary structures of these enzymes, and to obtain recombinant enzymes free from mutual contamination, we cloned the genes encoding α-1,3/4-fucosidase and lacto-*N*-biosidase of *Streptomyces* sp. strain 142. Chromosomal DNA was digested with restriction endonucleases, size-fractionated on agarose gel, and then treated by Southern hybridization with oligonucleotide probes, which were designed on the basis of partial sequences of the proteins. The genes encoding α-1,3/4-fucosidase and lacto-*N*-biosidase were cloned from the size-fractionated restriction fragments by colony hybridization. Both genes were sequenced and expressed in *Escherichia coli*. The open reading frame encoding α-1,3/4-fucosidase had 1689 bp and coded for a protein of 563 amino acids, and the open reading frame encoding lacto-*N*-biosidase had 1917 bp and coded for a protein of 639 amino acids. The recombinant enzymes were not contaminated with each other and had the same substrate specificities as the native enzymes.

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S3

Novel Approach for the Interaction Analysis between Sugar Chains and Carbohydrate Recognizing Molecules using a Biosensor Based on Surface Plasmon Resonance

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Recently, a biosensor based on surface plasmon resonance was developed. By using this technique, we have developed a new method for analysing the interaction between lectins and biotin-derivatized oligosaccharides. We also applied this method to characterize the structure of the sugar chains.

The complex type asialo-bi, tri and tetraantennary oligosaccharides were quantitatively converted into their biotin derivatives by incubating them with 6-(D-biotinyl)-aminohexanoyl hydrazide. This method was also applicable to sialyl sugar chains without any removal of sialic acid. The reaction mixture could be directly injected on to the streptavidin pre-immobilized surface of the sensor chip without any purification because of its fairly low reagent:carbohydrate molar ratio. The required amounts of sugar chains for the interaction analysis by this method were as low as 1 pmol. The binding specificities of *Sambucus sieboldiana* lectin, *Maackia amurensis* lectin, *Ricinus communis* agglutinin-120 (RCA_{120}) and Concanavalin A were rapidly determined qualitatively by this method. Conversely, the existences of terminal sialic acid, galactose, and *N*-acetylglucosamine were easily detected by measuring the interaction with those lectins. The structural information on the linkage of sialic acid and the branch numbers of oligosaccharides could be also determined. Furthermore, kinetic analysis of the interaction between RCA_{120} and the complex type asialo-bi, tri and tetraantennary oligosaccharides revealed that both the association rate constant and the dissociation rate constant (K_{diss}) were reduced by increasing the number of terminal galactosyl residues. These results suggested that kinetic parameters for the interaction could provide useful information for structural analysis.

S3

Structural Characterization of the *N*-glycans of Recombinant Human Factor IX

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Factor IX (FIX) is a single-chain glycoprotein which circulates in plasma as a zymogen of a serine protease. A deficiency in FIX, or functional defect in the FIX glycoprotein, is related to the bleeding disorder haemophilia B. During activation in the coagulation pathway by factor XIa or factor VIIa/tissue factor complex, FIX is cleaved at Arg^{145}/Ala^{146} and Arg^{180}/Val^{181} to yield activated FIX (FIXa). Removal of this activation peptide results in a two-chain molecule in which the C-terminal serine protease domain is disulfide bonded to the N-terminal γ-carboxyglutamic acid and epidermal growth factor-like domains. Whilst the activation peptide is not a known component of the coagulation cascade, post-translational modifications of the activation peptide, or the regions flanking it, may be important in modulating or regulating activation.

Recombinant human FIX (rhFIX) contains two consensus sequences for N-glycosylation both within the activation peptide, Asn^{157} and Asn^{167}, and both are occupied. Analysis of the PNGaseF-released N-glycans by high pH anion exchange chromatography (HPAE) illustrates the presence of over twenty neutral to tetrasialyl complex N-glycans. To further assist in the identification of the HPAE-separated glycans, we have been exploring the use of negative ion electrospray mass spectrometry (ES/MS). Since HPAE chromatographic eluents are incompatible with ES/MS, an on-line anion micromembrane suppressor has been used to desalt samples prior to mass spectrometric analysis. Based on the combination of the HPAE and ES/MS data, the major N-glycan from rhFIX is proposed to be a tetrasialyl tetraantennary structure with a single fucose. Less highly branched glycans and glycans containing poly-*N*-acetyllactosamine repeat units are present in less abundance.

S3

Construction and Characterization of N-Linked Oligosaccharide Libraries From Ovalbumin and Soybean Agglutinin

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Construction of libraries of different N-linked oligosaccharides may greatly advance the testing for biological activity and function of oligosaccharides. The isolation of a range of N-linked oligosaccharides from glycoproteins is one strategy that can be used to create an oligosaccharide library. The isolation and characterization of N-linked oligosaccharides from ovalbumin and soybean agglutinin (SBA) are described. Reducing oligosaccharides released from the protein were converted into their glycosylamines and then coupled with t-BOC-tyrosine *N*-hydroxysuccinimide ester to form the β-tryosinamide derivatives. Removal of the t-BOC group facilitated the RP-HPLC separation of oligosaccharides and in the case of ovalbumin fifteen oligosaccharides were isolated. Ten major components were obtained in amounts of one μmol or greater. Structural analysis by ^{1}H-NMR and FAB-MS showed the purified oligosaccharides had a range of structures including high mannose,

bisecting, and complex types. SBA produced a single oligosaccharide, Man9, which upon incubation with mannosidase gave an array of high mannose oligosaccharides.

The development of tyrosinamide oligosaccharide derivatives has allowed the purification of multiple oligosaccharide structures from glycoproteins. The presence of the tyrosine also allows the attachment of probes which enables these oligosaccharides to be used directly in biological assays.

S3

Equilibrium and Kinetic Parameters of the Interaction of Peroxidase Conjugate with Strophantine of Differing Composition with Antibodies Against Peroxidase

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The horseradish conjugates with strophantine containing 1, 2 and 3 glycoside molecule (HRP-STR) interaction with polyvalent antibodies against peroxidase (AntiHRP) has been studied by homogeneous enzyme-immunoassay.

The dissociation constants (K_d) of immunocomplexes [HRP-STR:AntiHRP] increased by increasing the strophantine molecule number in conjugates, and the rate constants of their formation decreased by increasing the modification degree of the HRP. The triple complex of the HRP-$(STR)_2$ conjugate with antibodies AntiHRP and AntiSTR is characterized by the highest K_d. The equilibrium and kinetic parameters obtained for an interaction of the HRP-$(STR)_n$ with the AntiHRP clearly reflected the variations in mass, volume and conformation of antigen at its modification.

S3

Simple Synthesis of Fluorescent Substrates for a Capillary Electrophoresis Based Assay of Glycosidases and Glycosyl-Transferases Using Commercially Available Aminophenyl-Glycosides

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The examination of glycosidase and glycosyltransferase enzymes often requires very sensitive detection methods. Many assays involving radioactivity, fluorescent and chromogenic substrates have been described. We have been developing substrates for use in assays based on capillary electrophoresis (CE) using a Beckman P/ACE with Laser induced fluorescence detection (LIF). Synthesis of fluorogenic carbohydrate substrates is normally time consuming and relatively difficult for the non-specialist laboratory. We have found the commercially available aminophenyl-glycosides provide a reasonable starting material for the production of carboxyfluoroscein labelled compounds which are very sensitive substrates for detection of a variety of glycosidases and glycosyltransferases when using CE-LIF detection.

A variety of aminophenylglycosides were labelled, and purified using a combination of thin layer chromatography and reversed phase HPLC so that each compound was a single peak on the electropherograms. The sensitivity of detection was in the range of $5 \times 10^5 - 1 \times 10^6$ molecules of reaction product (0.1–0.2 amol). These substrates could also be used to determine kinetic parameters.

S3

A New Method for Methylation Analysis by Using a Simultaneous Reaction for Degradation and Derivatization for HPLC

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To avoid complicated treatment of methylation analysis for GLC, a convenient and time-saving procedure for methylation analysis was developed using HPLC instead of the traditional GLC procedure.

Per-O-methylated oligo- and polysaccharides are degraded to their constituent monosaccharides and derivatized for UV-detection with p-nitrobenzoic anhydride simultaneously. Then the derivatives of constituent monosaccharides are analysed by HPLC.

S3

Capillary Zone Electrophoresis of Oligosaccharides Derivatized with Various Amine-bearing Derivatives of Aromatic Sulfonic Acids

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Amine-bearing derivatives of aromatic sulfonic acids are particularly appealing for pre-column derivatization in the analysis of oligosaccharides by capillary electrophoresis. They provide carbohydrates with both fluorescence detectability, as well as an overall negative charge over a wide pH range. The effect of degree of charge on speed of analysis and resolution of malto-oligosaccharides derivatized with different mono-, di- and trisulfonic acids was studied. The relationship between electrophoretic mobility and molecular structure was also investigated for differently derivatized oligosaccharides. The presence of a strong charge on sulfonate groups under both acidic and alkaline conditions allowed the use of variously composed buffers, exploiting different properties of derivatized oligosaccharides for their separation. This enabled us to compile multi-dimensional maps of relative migration times for N-linked oligosaccharides cleaved from various glycoproteins.

S4. NEW STRUCTURES OF N-LINKED AND O-LINKED CARBOHYDRATES

Poster presentations only

S4

Carbohydrate Sequencing of an N-Glycosylation Site in Recombinant Chimeric BR96 Monoclonal Antibody Using the Reagent-Array Analysis Method

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Chimeric BR96 is a monoclonal antibody produced by homologous recombination which binds to the Lewis Y antigen found in the surface of various human carcinomas. Each of the heavy chains of human immunoglobulin G (IgG) contains an asparagine-linked (N-linked) oligosaccharide in its Fc portion (Clamp *et al.*, 1964). The structure of the primary oligosaccharide present on one lot of recombinant chimeric BR96 was identified by the reagent-array analysis method (RAAM) and confirmed by monosaccharide compositional analysis and MALDI-TOF. The oligosaccharides on Chimeric BR96 monoclonal antibody were released with hydrazine using the Oxford Glycosystems GlycoPrep 1000. The free oligosaccharides were reduced and radiolabelled using sodium borotritide. The oligosaccharides were then purified by size exclusion chromatography through a P-4 column and separated peaks were collected. The primary peak was sequenced using the reagent array analysis method (RAAM) (Edge *et al.*, 1992). RAAM involves dividing the purified oligosaccharide into nine different aliquots. Each of the aliquots is then incubated with a different pool of exoglycosidases containing specific enzymes (an enzyme array). After incubation, the aliquots are combined and separated through a P-4 column by hydrodynamic volume. The resultant 'fingerprint' will be specific for each oligosaccharide. The structure was confirmed by monosaccharide compositional analysis and MALDI-TOF mass spectral analysis.

S4

Novel Oligosaccharide Structures are Indicated in Rat Brain

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Increasing evidence shows that complex oligosaccharides play important roles in recognition processes. The brain, by virtue of its enormous cellular and structural complexity, is an ideal tissue for candidate novel glycans within its oligosaccharide repertoire.

A tissue glycan library was prepared from a defatted preparation of rat brain by the method of anhydrous hydrazinolysis. Bio-Gel P4 gel permeation chromatography was used to separate neutral oligosaccharides (retarded) from acidic (voided). Most neutral glycans showed N-linked characteristics, with hydrodynamic volumes between 8.6 glucose units (GU) and 24 GU. Less abundant were neutral glycans in the range 3–8.6 GU. Monosaccharide compositional analyses of samples from the two glucose unit regions 3.5–5 GU and 6–8 GU were carried out by GC-MS of the trimethylsilyl(TMS)-1-*O*-methyl glycosides. These indicated the presence of mannose and glucose as major hexose constituents, and also the presence of the pentose, xylose. Matrix assisted laser desorption/ionization mass spectrometry of these two samples of 3.5–5 GU and 6–8 GU suggested that there were novel isobaric monosaccharide compositions within the 500–1400 Da range containing not only hexose, *N*-acetylhexosamine and deoxyhexose sugars but also showing the monosaccharide, pentose. It is already known that oligosaccharides are present in the brain carrying, unusually, an O-linked reducing terminal mannose [1]. The present results indicate further novel biosynthetic routes on these and related structures.

1. Krusius T, Finne J, Margolis RK, Margolis RU (1986) *J Biol Chem* **261**: 8237–42.

S4

Novel Structures and Location of N-Linked Sugar Chains in *Aspergillus niger* Crystalline α-Glucosidase

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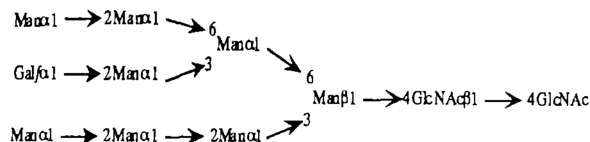
An α-glucosidase was crystallized from a commerical enzyme source (Transglucosidase Amano). The *A. niger* α-glucosidase (ANG) is composed of two subunits, P1 (MW, 3.3×10^4) and P2 (MW, 9.8×10^4) which are separable only by RP-HPLC. ANG is a glycoprotein containing 25.5% carbohydrate [1]. There are 24 modified amino acids in P1 and P2, which cannot be identified by Edman's method [2]. Fifteen of them were estimated to be N-glycosylated, because each sequence showed -Asn-X-Ser/Thr-, except for Ser-296 in P2: the Asn was deduced from the nucleotide sequence, and X was an optional amino acid. ANG was treated with N-glycosidase F, and seven oligosaccharide fractions were isolated by HPLC; one of them consisted of two components. The structures of the oligosaccharides were determined by ^{1}H-NMR and compositional analysis. Three structures of the sugar chains were identified to be $Man_8GlcNAc_2$, $Man_9GlcNAc_2$ and $GlcMan_9GlcNAc_2$. Each of the other four oligosaccharides contains an α-D-galactofuranosyl residue (Gal*f*) linked to Man *via* an α-1,2-linkage. The structures were determined to be Gal*f*$Man_5GlcNAc_2$, Gal*f*$Man_6GlcNAc_2$, Gal*f*$Man_7GlcNAc_2$, and Gal*f*Man_8-$GlcNAc_2$. Another structure remains unidentified. The position of linkage of Gal*f* residue in these sugar chains is identical with the oligosaccharides, Gal*f*$Man_4GlcNAc_2$ and Gal*f*Man_5-$GlcNAc_2$, first isolated from an α-galactosidase of the same origin [3]. Three sugar chains having Gal*f*, except for Gal*f*-$Man_5GlcNAc_2$, are novel structures. A structure of them, Gal*f*Man_8NAc_2, is as follows:



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2. Kimura A *et al*. (1992) *Biosci Biotech Biochem* **56**: 1368.
3. Takayanagi T *et al*. (1992) *Glycoconjugate J* **9**, 229.

S4

Unusual Oligosaccharides from Bovine Lung: N-Linked Glycosaminoglycans

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Glycosaminoglycan chains are thought to be attached to proteins almost exclusively via O-linkages. We previously reported evidence for small quantities of 'N-linked glycosaminoglycans' in bovine pulmonary artery endothelial cells [1]. Since a major fraction of the intact lung consists of endothelial cells we reasoned that bovine lung might be a rich source of similar molecules. Here, we describe the isolation and characterization of a collection of these unusual N-linked glycosaminoglycan structures from bovine lung acetone powder. The molecules are specifically released from proteins by Peptide:N-Glycosidase-F and account for ~10% of the released oligosaccharides. The specificity of the enzyme and the identification of *N*-acetylglucosamine at the reducing end clearly establish the presence of an N-linked core structure in these molecules. Treatments with heparinase, heparitinase, chondroitinases AC and ABC, and keratanase show that heparin/heparan sulfate, chondroitin sulfate, and keratan sulfate chains are attached to the core region. Some of these N-linked glycosaminoglycan structures possess at least two structurally distinct GAG species, e.g. heparin and chondroitin sulfate, attached to a single N-linked core. Interestingly, compositional analysis of these N-linked glycosaminoglycans indicates that xylose is present; however, the xylose moieties are not located at the reducing end as is typically found in O-linked GAGs. Presently, we are concentrating our efforts on establishing the linkage structure of the core region in order to identify the attachment site between the GAG chains and the core region.

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S4

Sialoglycoproteins of Nucleated (Chicken) Erythrocytes

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Glycophorins of human and some animal erythrocytes have been widely studied. However, there are no data on glycophorins of nucleated avian erythrocytes, most probably due to the more complex isolation procedure. Therefore, glycophorins of chicken erythrocytes were isolated, purified and characterized by SDS-PAGE, lectinoblotting and carbohydrate analysis. Electrophoretic fractionation of the crude membrane phenol-water extract, or of purified glycophorins, revealed three PAS-positive bands migrating as 70, 45 and 23 kDa molecules (the M_r was determined by comparison with the electrophoretic mobility of human glycophorins, taking into account their approximate MWs calculated from the primary structures). Carbohydrate composition of chicken glycophorins and methylation analysis of their β-elimination products indicated that the major O-linked structures are di- and monosialylated Gal1-3GalNAc- chains. The complex-type N-linked glycans were also present, including the biantennary chains with bisecting GlcNAc residue, and tri- or tetraantennary chains. The binding patterns of peanut agglutinin (PNA, anti-TF) and *Vicia villosa* lectin (VVL, anti-Tn) to chicken glycophorins in the blots confirmed the structure of O-glycans. All glycophorin bands showed the binding of PNA in desialylated blots only, and the binding of VVL in desialylated/Smith-degraded blots. Moreover, both lectins detected an additional 54 kDa band with the same lectin-binding properties. Interestingly, VVL also showed a weak binding to 70 and 45 kDa bands in the desialylated blots. Re-electrophoresis of the individual glycophorin species eluted from the gel suggested that 45 and 23 kDa bands are (at least partly) a dimer and monomer of the same glycoprotein, while the 70 kDa band represents a monomer of another glycoprotein. The relatively high M_r of this glycoprotein (over 100 000 when compared with conventional protein markers) raised the possibility that nucleated erythrocytes may contain leukosialin in addition to glycophorins. To find evidence for this suggestion, the preparation of rabbit antisera against individual electrophoretic chicken sialoglycoprotein species and immunochemical analysis of these species are in progress.

S4

Characterization of Oligosaccharide Structures of α_1-DSPA Expressed in Chinese Hamster Ovary-Cells

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The glycoprotein α_1-DSPA (*Desmodus rotundus* salivary plasminogen activator) is a plasminogen activator derived from the vampire bat *desmodus rotundus*. In contrast to other plasminogen activators, α_1-DSPA reveals strict fibrin specificity, a desirable attribute for thrombolytic therapy.

We investigated the unknown carbohydrate moiety of the two individual N-glycosylation sites of α_1-DSPA expressed by CHO-cells. Due to the wide structural homology to t-PA (tissue-type plasminogen activator) a similar glycosylation of α_1-DSPA was expected. α_1-DSPA was reduced, carboxymethylated and digested with trypsin. Peptides were separated by reversed phase HPLC. Glycopeptides were identified by monosaccharide analysis using anion exchange HPLC with pulsed amperometric detection (HPAEC-PAD) and by N-terminal sequencing. Oligosaccharides, enzymatically released by PNGase F, were separated by anion exchange chromatography according to their number of charges. Mainly neutral, monocharged and some bicharged glycans were detected. Separation of desialylated glycans by HPAEC-PAD revealed up to 10 different oligosaccharides for each glycosylation site. Analysis of individual oligosaccharides comprised mass-determination by MALDI-TOF-MS (matrix assisted laser desorption ionization time of flight mass spectrometry), monosaccharide analysis by HPAEC-PAD and linkage analysis by GC-MS (gas chromatography-mass spectrometry). For sequencing, glycans were fluorescence labelled with 2-aminobenzamide, digested by several mixtures of exoglycosidases and applied to gelchromato-

graphy. Computer assisted analysis suggested a structure of the original glycan. Summarizing all results, glycosylation site 1 was found to contain exclusively complex type oligosaccharides with bi, tri 224, tri 226 branched and tetraantennary structures, which were almost completely core fucosylated. In contrast to t-PA, glycosylation at Asn 117 contained not only high mannose and hybrid structures but complex type glycans. Main compound was the biantennary complex type sugar.

S4

Synthesis and Structure of Human Salivary Mucin O-Linked Glycopeptides

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Human salivary mucins protect the oral tissues by providing a physical barrier to environmental agents. They possess visco-elastic properties essential for lubrication and participate in the modulation of the oral flora. Chemical synthesis of salivary mucin (MG2) O-linked glycopeptides were undertaken to delineate O-glycosylation patterns which are important in mucin's biological activity and to study the effect of the carbohydrate moiety on the peptide backbone conformation. The 23 amino acid tandem repeat sequence of MG2 was used as a template to design mucin analogues. Two glycopeptides, APPET*T*AAP-OMe (*T* = glycosylated Thr) and PAPPS*S*SAP-OMe (*S* = glycosylated Ser) were prepared by solid phase synthesis using both Fmoc and t-Boc strategies. Glycosylated derivatives, Fmoc-Thr-[Ac_3-α-D-GalpN_3)]-OPfp and Fmoc-Ser-[Ac_3-α-D-GalpN_3)]-OPfp, were synthesized following the reported procedures and then incorporated into the tandem repeat to obtain glycopeptides having carbohydrate moiety on the desired Thr or Ser residues. The azide reduction was performed with thioacetic acid on the polymer bound glycopeptides. Corresponding apopeptides were also synthesized for comparison studies. Cleavage from the resin, deprotection and purification of the glycopeptides and apopeptides were carried out and their subsequent structural characterization was achieved by ^{1}H-NMR.

This work was supported by USPHS Grant DE07585.

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S4

Modification of O-Fucose Moieties with a β-Linked Glucose in CHO Cells

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Several serum proteins have recently been reported to be modified with an O-linked fucose at conserved sites in EGF domains. We are examining this form of glycosylation on endogenous proteins in CHO cells. A portion of the [^{3}H]-fucose metabolically incorporated into macromolecular material in these cells can be released by alkali-induced β-elimination. The β-eliminated material exists as two species. The major species corresponds to fucitol, and the minor species appears to be fucitol modified with a β-linked glucose. Previous workers [1] demonstrated the presence of Glcβ1,3Fuc-O-Ser/Thr amino acid glycosides in extracts of several cell types, including rat liver, over a decade ago. We believe that we have identified similar structures in CHO cells. Other workers have shown the presence of an O-fucose containing tetrasaccharide (Siaα2,6Galβ1,4GlcNAcβ1,3Fuc-O-Ser^{61}) on human factor IX [2]. It is not clear yet whether CHO cells also synthesize a similar tetrasaccharide. We are currently examining whether the Glcβ1,3Fuc disaccharide modification exists on a discrete subset of proteins modified with O-fucose or if it occurs at low levels on all O-fucose proteins. In addition, we are beginning studies on the novel enzymes involved in the O-fucose pathway.

This work was supported by NIH Grant GM 48666.

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2. Harris *et al.* (1993) *Biochemistry* **32**: 6539.

S4

Glycosyl Imidates: A Stereoselective, Facile Synthesis of Oligomers as Allyl Glycosides Related to Antigenic Determinants of Bacterial O-Lipopolysaccharides (LPS) for Conversion into Neoglycoconjugates (NGCs)

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Study in the field of complex oligosaccharides related to bacterial O-LPS has proven to be a principal part of many biological and medical studies in recent years. The synthesis of oligosaccharides in combination with the strategies for the covalent attachment of these hapten to a solid support permits the use of these compounds as substitutes for polysaccharides of bacterial origin in several serological tests and eventually as vaccines.

Escherichia Coli (*E. coli*) and *Salmonella* serotype (C_2 and C_3), the typical opportunistic bacilli which function as important human pathogens have been chosen for the present study. The initial steps for the synthesis of the immunodominant regions of the above potential antigen involved the synthesis of derivatives of sugars viz. D-Glc; D-Man and L-Rha; involving modern regio- and stereoselective protecting group strategies. Special care was taken with the substitution of various hydroxyl groups in the glycosyl derivatives as they influence the reactivity of the various reactants in glycosidation reactions. Thus, α-L-Rha-$(1 \rightarrow 3)$-α-D-Manp and α-L-Rha-$(1 \rightarrow 2)$-α-D-Manp were synthesized with the objective of gaining a detailed insight into the structural requirement for studying the pharmacological parameters of the biological repeating units of bacterial O-LPS viz. *E. coli* and *Salmonella* serotype (C_2 and C_3) respectively using the trichloroacetimidate method of glycosidation. Likewise, α-L-Rha-$(1 \rightarrow 6)$-α-D-Manp-$(4 \leftarrow 1)$-α-L-Rha were also synthesized. The key feature in glycosylation path involved stereochemical activation of a 2,3,4 tri-*O*-acetyl-α-L-rhamnopyranosyl trichloroacetimidate donor intermediate in the presence of a Lewis acid catalyst such as BF_3-etherate which with the help of an *O*-acyl group at C-2 complied 1,2-trans-O-interglycosidic linkages exclusively. The various di- and trisaccharides were synthesized in the form of allyl glycosides for the subsequent covalent attachment to protein such as bovine serum albumin (BSA) via ozonolysis and reductive amination. Such compounds are expected to be potentially useful in immunological and immunochemical studies of these carbohydrate antigens.

S4

Structure of the Main Saccharide Chain of the Acrosome Reaction-Inducing Substance (ARIS) in the Egg-Jelly of Starfish, *Asterias amurensis*

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The acrosome reaction (AR) is a prerequisite for fertilization in various animals including mammals. In starfish, three components of the egg jelly are responsible for triggering the AR, of which a sulfated glycoprotein named acrosome reaction-inducing substance (ARIS) is the key signal molecule. The activity of ARIS is mostly due to sulfated sugar chains.

An actinase digest of ARIS from *A. amurensis* was purified by ion exchange chromatography on DEAE Toyopearl 650M. The major fraction consisting sulfated sugar chains was biologically active at a concentration of $5\,\mu g\,ml^{-1}$ and was further studied. The structure of the saccharide chain was found to be a linear polymer of $1 \rightarrow 4$-linked β-xylopyranosyl,$1 \rightarrow 3$-linked α-galactopyranosyl,$1 \rightarrow 4$-linked α-fucopyranosyl and 3-linked α-fucopyranosyl with 4-O-sulfation in a molar ratio of 1:1:1:2 as its repeating structural unit using chemical analysis together with NMR spectroscopy.

S4

Sulfation of Human Respiratory Mucins

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Abnormal mucin sulfation has been observed in respiratory mucins from patients suffering from cystic fibrosis (CF). In order to define the sulfotransferases involved in the biosynthesis of human respiratory mucins and their possible abnormalities in CF, it is important to determine the precise localization of sulfate on carbohydrate chains from CF and non-CF respiratory mucins.

The structural determination of sulfated oligosaccharides-alditols from human respiratory mucins has shown that sulfation may occur either on the C-3 of a terminal galactose (Gal) residue or on the C6 of an N-acetylglucosamine residue. These data suggest that the sulfation of human respiratory mucins involves at least two sulfotransferases.

A Gal-3-O-sulfotransferase activity able to transfer a sulfate group from PAPS on to methyl galactosides or terminal galactose residues of carbohydrate chains from human respiratory mucins has been found in microsomal fractions prepared from human respiratory mucosa. The reaction products were identified by high performance anion-exchange chromatography.

Using methyl β-galactoside as a substrate, the optimum activity was obtained with 0.1% Triton X-100, 30 mM NaF, 20 mM Mn^{2+} and 10 mM AMP in a 30 mM MES buffer at pH 6.1.

This enzyme had more affinity for carbohydrate chains with a terminal Galβ1-4GlcNAc sequence than for methyl β-galactoside.

This work has been supported by the Association Française de Lutte contre la Mucoviscidose.

S4

Structure and Function of Sulfated Oligosaccharides Derived from Acrosome Reaction-inducing Substance (ARIS) of Starfish

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Sperm of most animals must undergo acrosome reaction, an exocytic event, prior to cell membrane fusion with an egg during fertilization. Acrosome reaction is induced by substance(s) in the extracellular egg coat. However the molecular nature of the molecule(s) triggering the acrosome reaction is not characterized in most animals but has been in starfish. In the starfish, *Asterias amurensis*, acrosome reaction is induced by three components; sulfated glycoprotein of an extremely large molecular size, a group of sulfated steroid saponins and a group of peptides. Among them the glycoprotein plays the main role in inducing acrosome reaction and is named ARIS. Pronase digest of ARIS (P-ARIS) shows the full activity of ARIS, indicating the importance of sugar chains in the induction of the acrosome reaction.

P-ARIS mainly consists of -4Xyl1-, -3Gal1-, -4Fuc1-, -3Fuc1- and -3,4Fuc1-, which were determined by methylation analysis. Mild acid hydrolysis (10 mM H_2SO_4, 100 °C, 1 h) of P-ARIS liberated several oligosaccharides. The major one was elucidated by compositional analysis and FAB-MS. To overcome difficulties in determining anomers and the position of the sulfate group several predicted saccharides were synthesized and compared with a natural one. It was determined to be Xylβ1-3Galα1-3(4SO_3^-)Fuc. The precise structures of monosulfated tetra- and pentasaccharide are now under investigation.

Egg jelly induces acrosome reaction in sea water whereas ARIS alone is enough in high Ca^{2+} or high pH sea water. Treatment of sperm with only ARIS in sea water insensitizes them for the induction of acrosome reaction by jelly solution. Most of ARIS-derived and synthetic sulfated oligosaccharides insensitized spern but to different degrees.

S4

Detailed Structural Analysis of a Novel, Specific O-Linked Glycan from Secreted *Flavobacterium meningosepticum* Glycoproteins with Asp-Ser* and Asp-Thr*-Thr Consensus Sites

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Prokaryotic extracellular glycoproteins with O-linked oligosaccharides at specific consensus sites have never been described. We report a new type of O-linked oligosaccharide on several proteins secreted by the Gram-negative bacterium *Flavobacterium meningosepticum*, including Endo F_2 (3 sites), Endo F_3 (1 site) and a P40 protease (1 site). The oligosaccharide moiety is covalently attached via a mannose residue to a serine or threonine at consensus sites corresponding to Asp-Ser* or Asp-Thr*-Thr. Preliminary characterization by mass spectroscopy revealed an oligosaccharide of 1244 Da at each of

the proposed glycosylation sites. Collision-association dissocation analysis showed a characteristic daughter ion series of *m/z* 218, 394, and 556, indicative of a common *Flavobacterium* oligosaccharide. The singly branched structure contained seven residues including three different uronyl analogues, a methylated rhamnose and mannose, a glucose, and a reducing terminal mannose. One pyranose ring forms were detected. The proposed structure was supported by a combination of isotopic labelling, composition and methylation analysis, and the preparation of several chemical analogues and derivatives with each product evaluated by MS and CID.

S4

Characterization of Sulfated N-glycans from Recombinant Human Erythropoietin Expressed in Chinese Hamster Ovary Cells

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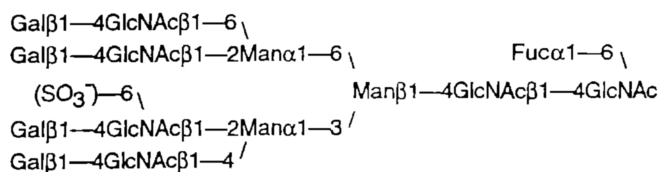
A portion of the N-glycans present on recombinant human erythropoietin (rHuEPO) expressed in Chinese hamster ovary cells have been found to carry a sialidase resistant charge that was tentatively identified as a sulfate ester based on a variety of analysis including metabolic labelling with [^{35}S]-sulfate. While the sulfates appear to be distributed over all species of N-glycans typically present on rHuEPO, a major fraction (c.a. 56% of the sulfated species) was isolated and initially characterized as a core fucosylated tetra-antennary oligosaccharide with one sulfate residue. Examination of this fraction by ^{1}H-NMR revealed the following structure:



Based on these findings a scheme involving digestion with a variety of exoglycosidases, chemical desulfation by mild methanolysis, and BioGel P4 chromatography was developed and used to provide evidence for a similar site of attachment on the other mono-sulfated N-glycans from rHuEPO.

S4

Differential *N*-Acetylgalactosaminylation of Bovine and Human Mammary Epithelial Glycoproteins

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We have previously reported that most bovine milk fat globule membrane (MFGM) glycoproteins contain N-linked sugar chains with GalNAcβ1 → 4GlcNAc structure [1]. The *N*-acetylgalactosaminylated sugar chains were not detected in glycoproteins of the bovine mammary epithelial primary cultured cells which do not produce any type of caseins, suggesting that the expression of the disaccharide structure is associated with the functional development of the tissue. In contrast, no human MFGM glycoproteins were shown to contain the *N*-acetylgalactosaminylated sugar chains. However, lectin blot and structural analyses of *N*-linked sugar chains from MRK-nu-1 cells established from a human breast tumour revealed that many glycoproteins contain the sugar chains with GalNAc-β1 → 4GlcNAc structure, to which WFA lectin binds. Lectin blot analyses of glycoprotein samples from normal human breast tissue and human breast cancer showed that both samples contain WFA-reactive bands though the number of WFA-positive bands is smaller in the normal sample. Since no WFA-positive bands were detected in the human MFGM preparation [1] and since the normal breast tissue is rich in adipocytes, the WFA-positive glycoproteins may be derived from adipocytes in the normal tissue. These results indicate that the differential *N*-acetylgalactosaminylation of bovine and human mammary epithelial glycoproteins may be due to the differential regulation of *N*-acetylgalactosaminyltransferase activity in these animals.

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S4

A Precise Structural Analysis of Fertilization-associated Carbohydrate-rich Glycopolyprotein from the Eggs of *Tribolodon hakonensis* (Dace). Tetraantennary N-glycans with highly sulfated poly-*N*-acetyllactosaminyl chains

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In the last several years we have been involved in the detailed studies of structure, biosynthesis, and physiological function of cortical alveolar-derived carbohydrate-rich glycopolyproteins (hyosophorin) originated from teleost fish eggs. In this Symposium, we present the precise structural analysis of a novel type of bulky tetraantennary N-linked glycan ($\langle M_r \rangle$ = 10 K) present in the hyosophorin molecules isolated from the unfertilized eggs of *Tribolodon hakonensis* (a dace). By methylation analysis, endo-β-galactosidase digestion, hydrazinolysis-nitrous acid deamination, mild methanolysis, FAB-MS spectrometry, and ^{1}H-NMR spectroscopy, the glycan chains were shown to have the skeletal poly-*N*-acetyllactosamine sequences which were frequently branched at the Gal residues [GlcNAcβ1 → 3(Galβ1 → 4)Galβ1 → 4] and 6-*O*-sulfated at multiple numbers of GlcNAc residues. The possible biological significance of the expression of such multiple numbers of sulfate groups on a single multiantennary glycan unit is discussed with special reference to the unique structures of hyosophorins from other fish species [1–3].

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S4

Characterization of Heparan Sulfates by ^{1}H-NMR and CE Analysis

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A study on the structure of porcine heparan sulfate using 500 MHz ^{1}H-NMR spectroscopy and capillary electrophoresis is presented. The distribution of heparan sulfate in porcine tissues varied considerably. Structural diversity exists between renal heparan sulfate and intestinal mucosal heparan sulfate. Quantitative and qualitative ^{1}H-NMR determination was acquired with particular attention to the proton signals of the O-sulfated, and N-sulfated carbon atoms. An equatorial proton attached to 2-O-sulfated carbon of iduronate residue was observed at 0.9 ppm downfield compared to that attached to unsulfated carbon. In contrast, an axial proton binding to N-sulfated carbon of the glucosamine residue was found at 0.7 ppm upfield compared to that of the N-acetylated glucosamine. One dimensional differential NOE experiments on an intact heparan sulfate chain was used to detect and identify minor signals in the spectra. A novel structural feature in renal medullary heparan sulfate was also demonstrated by both ^{1}H-NMR and capillary electrophoresis. Heparin lyase digestion of heparan sulfate followed by capillary electrophoresis also confirmed saccharide composition and structure. Both analytical methods showed the content of sulfate ester groups and of iduronic acid residues in each heparan sulfate isolated from different porcine organs. These results suggest that the biosynthesis, of heparan sulfate in different porcine tissue, is differentially controlled.

S4

Unusual Oligosaccharides from Bovine Lung: Carboxylic Acids on Desialylated N-linked Oligosaccharides

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We have previously shown that bovine pulmonary artery endothelial cells contain a diverse family of sulfated and sialylated anionic N-linked oligosaccharides [1]. This prompted us to characterize the 'library' of Peptide:N glycosidase F-released N-linked oligosaccharides from bovine lung, an abundant source of endothelial cells. Radiolabelled glycans of both low or high negative charges were prepared and partially characterized. The oligosaccharides with low anionic charge were studied by complementary techniques including: (i) monosaccharide compositional analysis; (ii) linkage analysis; (iii) sulfate content; (iv) lectin affinity chromatography; (v) charge composition analysis; and (vi) neutralization of sulfate, phosphate, and carboxylic acid groups. The N-linkage of the oligosaccharides was confirmed by the presence of only [^{3}H]N-acetylglucosaminitol in the NaB[^{3}H]$_4$-reduced molecules. Furthermore, the core of these structures indicates the expected tri-mannosyl chitobiose core, and suggest a prevalence of tri- or tetraantennary structures. Surprisingly, although a substantial amount of sialic acid was present, the majority (~65%) of remaining negative charge on the desialylated oligosaccharides could *not* be attributed to either phosphate or sulfate groups. However, most of these charges could be reversibly neutralized by various chemical treatments specific towards carboxylic acid groups, including methyl esterification. Furthermore, anionic charge profiling indicates the presence of multiple carboxylic acid groups on the same N-linked oligosaccharide, not due to sialic acids. Further studies are underway to identify the exact nature of these unusual anionic residues.

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S4

Sequence Analysis of N- and O-linked Carbohydrates in Human Urinary Bikunin

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Bikunin, which has protease inhibitory activity, is held together with heavy chains via a chondroitin sulfate, to form members of the inter-α-trypsin inhibitor family in human blood plasma. We have previously given evidence that low-sulfated chondroitin 4-sulfate (LSC), which is the major glycosaminoglycan in blood plasma, is covalently bonded to core-protein of bikunin. It contains the LSC and an N-linked oligosaccharide. By using the pyridylamino-method, we confirmed the structures of the N-linked oligosaccharides in human urinary bikunin: (±Neu5Ac)α2-6Galβ1-4GlcNAcβ1-2Manα1-3[(±Neu5Ac)α2-6 Galβ1-4GlcNAcβ1-2Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAc. Furthermore, we characterized the LSC from human urinary bikunin. With hyaluronidase SD (from *Streptococcus dysgalactiae*) digestion, ΔDi-0S and oligosaccharides that contain the linkage region were obtained from LSC. We revealed that the LSC is a unique type of the chondroitin sulfate having localization of 4-*O*-sulfo GalNAc residues around the linkage region based upon the structural analyses of the oligosaccharides. Recently, charge isomers of urinary bikunin have been found, and we then examined the structure of LSC from each isomer. It is worth noting that common linkage legions, GlcAβ1-3Gal(4S)β1-3Galβ1-4xyl, are followed by [Di-4S]$_3$, [Di-4S]$_4$, [Di-4S]$_5$ or [Di-4S]$_6$ (GlcAβ1-3GalNAc(4S)β1-4 is abbreviated as Di-4S), and then [GlcAβ1-3GalNAcβ1-4] units extend to the non-reducing end. We presume that the cross-link to heavy chains exists in the non-sulfated region of LSC, considering the steric hindrance from sulfate at the C-4 hydroxyl group.

S4

New Glycoconjugates Derived from Conventional Synthetic and Microwave Dielectric Heating-Mediated Techniques

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New methods for the preparation of several types of glycoconjugates have been explored. A series of glycoconjugates was derived from synthetic polymers, including poly(vinyl acetate) and poly(vinyl alcohol). Condensation of these substrates with mono-, di-, or oligosaccharides yielded new N-alkyl, acetal, or ester conjugates. A second series of glycoconjugates was

obtained by the coupling of O-carboxamide glucan precursors and various proteins under mild reaction conditions. As an alternative to conventional techniques, microwave dielectric heating-assisted reactions were also explored for the preparation of new lipophilic glycoconjugates.

*This work was conducted at the Pulp and Paper Research Centre and the Department of Chemistry, McGill University, 3420 University Street, Montreal, Quebec, H3A 2A7, Canada.

S5. NEW STRUCTURES OF LIPID-LINKED CARBOHYDRATES, INCLUDING ANCHORING LIPID GLYCANS

Poster presentations only

S5

A Novel Glycolipid of *Cryptococcus neoformans*

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Lipids were extracted from cell homogenates of fungus body using chloroform-methanol mixtures, and subjected to Folch's partition. Isolated glycolipids (GL-1 to GL-3) were obtained by repeated Iatrobeads column chromatography of lower phase lipids of Folch's partition.

GL-1 had the same Rf value on TLC with that of ceramide monohexoside obtained from ox brain. GL-2 and GL-3 showed a lower R_f value than that of GL-1. IR spectrum of GL-1 showed nearly the same absorptions with those of glycosylglyceroldialkylether. Sugar analysis as TMS-methyl glycoside revealed only glucose, and analysis as partially methylated alditol acetates showed the presence of Glc1-. ^{1}H-NMR indicated a β-configuration of glucose. From these results the structure of GL-1 was determined as 1,2-di-O-alkenyl-3-β(D-glucopyranosyl)-sn-glycerol. FAB-MS of GL-1 supported this structure. Main component of the alkenyl chain was hexadecene and octadecene. The position of the double bond has not yet been characterized. Structural studies on GL-2 and GL-3 are now in progress.

S5

Spirometosides: Glycosphingolipids Consisting of Novel Carbohydrate Structures with a Penultimate Glucose, Glcβ1-3Gal, from Parasite *Spirometra erinacei*

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Glycosphingolipids could be expected to be involved in the mediation of host-parasite interactions, as are functioning in bacterial and viral infection. Previously we isolated a novel fucosylated glycosphingolipid (SEGLx) from the parasite, *Spirometra erinacei* [1], the structure being determined to be Galβ1-4(Fucα1-3)Glcβ1-3Gal-Cer. In this study, we found a fucosyltetrahexosylceramide (Gal-SEGLx) having an additional galactose molecule attached to the reducing end galactose of SEGLx through a β1-6 linkage. In addition to cerebroside [2], we determined di- and trihexosylceramides, both of which consisted of a Glcβ1-3Gal structure, suggesting possible biosynthetic precursors of SEGLx and Gal-SEGLx. Thus, all membrane glycolipids of this parasite (cestode) have been found to consist of a new class of glycolipids. We here propose the term 'Spirometo series', which represents this new type of carbohydrate series, and 'Spirometosides' for glycolipids having the Spirometo carbohydrate structure.

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S5

Isolation and Structural Analysis of Phosphorylated Oligosaccharides from *Escherichia coli* J-5 Lipopolysaccharide

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Lipopolysaccharides (LPS) are responsible for many of the pathophysiological effects observed during infection with gram-negative pathogens. These can lead to septic shock and are responsible for more than 100 000 cases of death in the US yearly. One of the most promising concepts for the treatment of septic shock is passive immunization with antibodies which are directed against the conserved regions of LPS. The core-defective Rc-mutant *Escherichia coli* J-5 was claimed to be particularly suited to induce such antibodies. However, several monoclonal antibodies have been isolated, none of which was able to reduce mortality due to septic shock in clinical studies. Recently, a mAb (WN1 222-5) was isolated which was shown to be cross-reactive *in vitro* with all tested clinical isolates of *E. coli* and *Salmonella* and was cross-protective *in vivo*. Although WN1 222-5 was obtained after immunization with a more complex LPS (complete core) it recognized as a minimal structure phosphorylated LPS of *E. coli* J-5, whereas the recognition of larger oligosaccharides was independent from phosphate substitution. To investigate structural requirements for antibodies to be cross-protective against gram-negative pathogens, we isolated the complete phosphorylated carbohydrate backbones of *E. coli* J-5 LPS after deacylation by HPAE and determined their structures by one-dimensional ^{1}H-, ^{13}C- and ^{31}P-NMR, two-dimensional homo- and heteronuclear

NMR as well as NOESY. Full assignment of chemical shifts and coupling constants revealed a nonsaccharide, an octasaccharide and two heptasaccharides with up to four phosphorylation sites. The obtained results may give additional information about the biosynthesis of LPS.

S5

Minor Components of the Bovine Brain Ganglioside Mixture

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Three new minor components 1, 2, 3 covering about 0.02%, 0.12% and 0.08% of the total bovine brain ganglioside mixture, respectively, were characterized as: 1, Neuα2-3Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glcβ1-1Cer; 2, GalNAcβ1-4-(Neu5Acα2-3)Galβ1-3GalNAcβ1-4(Neu5Gcα2-3)Galβ1-4Glc-β1-1Cer; 3, GalNAcβ1-4(Neu5Gcα2-3)Galβ1-3GalNAcβ1-4-(Neu5Acα2-3)Galβ1-4Glcβ1-1Cer.

The purification of the three compounds was carried out starting with 50 g of the total ganglioside mixture and yielded 1 mg of pure 1 and 20 mg of a mixture of 2 and 3. The purification consisted of the following steps: *clostridium perfrigens* sialidase treatment of the ganglioside mixture (the three compounds were sialidase resistent), DEAE-sephadex column chromatography and silica gel column chromatography. The three gangliosides were characterized by ^{1}H and ^{13}C 1- and 2-D NMR spectroscopy. The structure of compounds 2 and 3 was confirmed by enzymatic hydrolysis using exosaminidase A and *clostridium perfrigens* sialidase in a combination of treatments. The released products were analysed by HPTLC using reference compounds.

S5

Extended Type-1 Chain Glycosphingolipids: Isolation and Structural Characterization of Sialyl Dimeric-Lewis[a]

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Sialyl-Le^{a} is a tumour-associated carbohydrate antigen based on the type-1 chain core and was originally described as a sialylated Le^{a}-active pentasaccharide defined by MAb 19-9 [1]. Recently, a series of novel sialyl-Le^{a} active gangliosides containing a combination of branched and linear type-1 and type-2 chain cores was isolated from human rectal adenocarcinoma [2]. In this study a sialyl-Le^{a} active glycosphingolipid containing a repetitive type-1 chain carbohydrate core was isolated from human colonic adenocarcinoma cell line Colo205. Glycosphingolipids with this novel core structure have only recently been described and to our knowledge have only been isolated as neutral glycolipids [3]. The sialyl-Le^{a} active ganglioside was purified by HPLC and preparative HPTLC and its structure elucidated by positive-ion FAB-MS and ^{1}H-NMR. The glycosphingolipid was found to be a sialyled difucosylated derivative of this novel carbohydrate core.

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3. Stroud M *et al*. (1993) 'Carbohydrate Antigens' ACS Symposium Series, **519**: 159.

S5

Rat Glioma Ganglioside GM3 Having 3-*O*-Acetylated Sphingenine

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A novel *O*-acetylated GM3 containing 3-*O*-acetyl(Ac)4-sphingenine was isolated with one having a nonacetylated base from transplanted rat glioma tissue. The presence and position of the Ac group was estimated by one- and two-dimensional proton nuclear magnetic resonance (NMR), and fast atom bombardment-mass spectrometries. The *O*-acetylated sphingenine, of which H-3 shifted to a lower-field (δ5.21) in the NMR spectrum, was neither detected in any neutral glycosphingolipids (GSLs) from glioma, nor in any GSLs from normal rat brain, indicating the occurrence of nonacetylation on these GSLs, and further suggesting that expression of *O*-Ac Cer on GM3 might be a tumour-associated phenomenon. The extent of the lipid-O-acetylation in glioma GM3 was estimated with integration of olefinic H-5 on sphingenine, of which a chemical shift was distinct between *O*-acetylated and nonacetylated GM3, to 35%. The *O*-Ac sialylparagloboside at the Cer was also obtained from the tissue as a minor ganglioside. In addition, the *O*-Ac GM3 showed higher immunological activity towards anti-melanoma antibody (M2590) in the presence of nonacetylated GM3 in complement-dependent liposome lysis than did nonacetylated or acetylated GM3 alone in the liposome, suggesting enhancement of immunological reactivity of the intact tumour cells by a small amount of *O*-Ac GM3.

S5

Novel Sphingolipids with Inositolphosphate-Containing Head Groups from Lugworm

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A family of sphingolipids with inositolphosphate-containing head groups referred to as 'Phytoglycolipids' and/or 'Mycoglycolipids' has been characterized in plants, fungi and protozoans, but not thus far in animals. Recently, we characterized two glycolipids containing an inositolphosphate residue from a marine animal. In this symposium, we describe the isolation and the molecular structures of two acidic glycolipids (provisionally named AGL_4 and AGL_5) from the whole tissue of the lugworm, *Tylorhynchus heterochaetus*. These acidic glycolipids were purified by QAE- and DEAE-Sephadex and silicic acid, and their structures were elucidated by compositional sugar analysis, hydrogen fluoride degradation, methylation analysis, periodate oxidation, negative ion mode FAB-MS spectrometry and ^{1}H-NMR spectroscopy to be as follows; AGL_4:Man(α1-6)Ino(1$\rightarrow$)-P-Cer, AGL_5:Man(α1-6)[Fuc(α1-3)]Ino(1$\rightarrow$)-P-Cer.

S5

Structural Characterization of a Sulfated GM1a, the Unique Sulfated Ganglioside from Rat Kidney

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Rat kidney contains a large variety of sulfated glycosphingolipids belonging to the ganglio-series and isoglobo-series [1] core structures. All have one or two sulfate esters at C-3 of Gal and/or GalNAc. In the present study, we isolated a novel glycosphingolipid (Kd), which was found to contain both a sulfate ester and a sialic acid in the molecule.

Kd was purified from rat kidney by FPLC with DEAE-Toyopearl and HPLC with Iatrobeads. 1-D ^{1}H-NMR, compositional and permethylation analyses showed that Kd has a Gg_4Cer core with 1 mol each of sulfate ester and NeuGc at C-3 of Gal. The major ceramide consisted of 24:0/t18:0, deduced from the compositional analysis and negative LSIMS [2]. Mild acid hydrolysis and solvolysis produced compounds which migrate similarly to Gg_4CerIV^3-sulfate (SM1b) and II^{3}NeuGcα-Gg_4Cer (GM1a), respectively, on TLC. The abundant ions characteristic for sulfated mono- and disaccharides in high-energy CID spectra were consistent with the structure at the non-reducing terminus, HSO_3-*O*-Hex-*O*-HexNAc- rather than the alternative structure, NeuGc-*O*-Hex-*O*-HexNAc. The 2-D DQF-COSY experiment further evidenced the presence of a 3-*O*-sulfated Gal in the molecule. From these results the complete structure of Kd was proposed to be HSO_3-3Galβ-3-GalNAcβ-4(NeuGcα-3)Galβ-4Glcβ-1Cer(II^{3}NeuGcα-Gg_4Cer-IV^3-sulfate). This is a novel class of sulfoglyolipid, a sulfate analogue of ganglioside.

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S5

Characterization of Four Novel Mono- and Di-*O*-Ac GM3s from Equine Erythrocytes

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Two novel mono-*O*-Ac GM3s containing 9-*O*-Ac NeuGc (9-*O*-Ac GM3) and containing 6-*O*-Ac Gal (6′-*O*-Ac GM3) as a mixture, and two novel di-*O*-Ac GM3s containing 4,9-di-*O*-Ac NeuGc (4,9-di-*O*-Ac GM3), and containing 4-*O*-Ac NeuGc and 6-*O*-Ac Gal (4,6′-di-*O*-Ac GM3) as a mixture were obtained from equine erythrocytes as the minor components. The structures were characterized by one- and two-dimensional proton nuclear magnetic resonance (NMR) and fast atom bombardment-mass spectrometry. The position of the *O*-Ac residue was identified by the lower field-shifts of the proton attached to the carbon bearing acetoxy group on sialic acid and/or galactose in the mono- and di-*O*-Ac GM3s, than those of the respective unacetylated GM3. To confirm these acetylated structures, GM3 and 4-*O*-Ac GM3 were chemically *O*-acetylated using ortho acetic acid, revealing several partially *O*-acetylated derivatives. Of the chemically acetylated GM3s, the NMR spectra of 9/6′-*O*-Ac GM3 and 4,9/4,6′-di-*O*-Ac GM3 showed identical chemical shifts of the sugar ring protons to those of natural Ac GM3s except for the intensities. Both the purified natural and synthesized di-*O*-Ac GM3s were not desialylated with neuraminidase, which extensively cleaves 9-*O*-Ac NeuGc, indicating that the reaction was inhibited by the presence of the 4-*O*-Ac group, whereas 9/6′-*O*-Ac GM3 gave LacCer and 6′-*O*-Ac LacCer.

S6. NEW STRUCTURES OF GLYCOCONJUGATES FROM PLANTS AND MICROBES

Poster presentations only

S6

Glycosylation of an Aspartic Proteinase from *Cynara carduncunlus* L.

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An aspartic proteinase present in the flowers of the cardoon *Cynara carduncunlus* L., is used in the production of cheese in Portugal [1]. The enzyme consists of two subunits of apparent molecular masses of 30 and 15 kDa with a N-glycosylation site each [2]. The carbohydrate accounts for 11% (by weight) of the protein weight. The glycosylation of the proteinase is of interest because it might modulate the enzyme's milk clotting and proteolytic activities as recently reported for the proteinase from *Mucor pusillus*, also used for cheese production [3]. Consequently, in the present work the structures of the glycans of the enzyme were studied.

Preliminary monosaccharide composition studies have shown that each subunit contains Man, Fuc, Gal, Glc and hexosamine residues [2]. Affinity blotting showed that both subunits are recognized by concanavalin A and *Tetragonolobus purpureas* lectin. Treatment with PNGase F under both denaturing and non-denaturing conditions deglycosylated only about 1% of the 30 kDa subunit. However, when the proteinase was chemically defucosylated, about 50% of both subunits could be deglycosylated with PNGase F. These results together suggest that the glycans of the proteinase are of the plant complex type with proximal α(1,3)-linked fucose.

The structures of the major oligosaccharides released by PNGase A from Pronase glycopeptides, based on Bio-Gel P-4 gel filtration chromatography and exoglycosidase sequencing

will be presented.
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S6

Identification of Novel Glycosylated Entities in the Secreted Protein and Cell Wall of Mycobacteria

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The detailed structural characterization on various cell wall, membrane associated and secreted glycoconjugates of mycobacteria continues to furnish new findings which challenge our current perception of their cell wall architecture and its potential roles in pathogenesis. All mycobacterial species are endowed with a mycolylarabinogalactan-peptidoglycan (mAGP) complex which constitutes the cell wall proper. Associated with the plasma membrane and somehow protruding through this cell wall are the phosphatidylinositol-anchored lipoarabinomannan (LAM) and the structurally related lipomannan (LM). In addition, there are cell wall associated, surface associated and secreted proteins, some of which have recently been shown to be glycosylated.

We present here our recent structural data on various mycobacterial glycosylated entities, as obtained through chemical/enzymatic digestions, chromatographic separation and mass spectrometric analysis in conjunction with further chemical derivatization. In support of earlier observation but at odds with current model for the mAGP, we now obtained firm chemical evidence for the presence of a non-N-acetylated galactosamine covalently linked to arabinan in the arabinogalactan. Endo-α1,6-manannase digestion on LAM and LM with and without further chemical/enzymatic manipulation allows us to define the structural motifs on the mannan cores of LAM/LM and their acylated and phosphorylated variants. We have also succeeded in obtaining further chemical and mass spectrometric evidence demonstrating the presence of protein O-glycosylation in mycobacteria. α-Mannosidase digestion on a series of glycopeptides from the secreted 50 kDa glycoprotein indicates that it is primarily O-mannosylated in at least three different sites.

S6

Characterization of a phosphocholine-containing Aminoglycoglycerolipid: Major Lipid Antigen of *Mycoplasma fermentans*

K. Matsuda[1], T. Taki[2], T. Kasama[3], I. Ishizuka[4], S. Handa[2] and N. Yamamoto[1]
[1] *Department of Microbiology,* [2] *Department of Biochemistry,*
[3] *Laboratory of Biomedical Analysis, Tokyo Medical and Dental University, Tokyo, Japan.*
[4] *Department of Biochemistry, Faculty of Medicine, Teikyo University, Tokyo, Japan.*

Mycoplasma fermentans is thought to be a pathogen of rheumatoid arthritis or cofactor of AIDS. We have demonstrated that phosphocholine-containing glycoglycerolipids (GGPLs, GGPL-I and GGPL-III) are major lipid components of *M. fermentans*. We reported the structure of GGPL-I as 6'-O-phosphocholine-α-glucopyranosyl-(1'-3)-1,2-diacyl-*sn*-glycerol [1]. In the previous study, the GGPL-III was purified by DEAE-Sephadex and repeated Iatrobeads column chromatographies. The purified GGPL-III was subjected to structural characterization by chemical staining on HPTLC-plate, FT-IR, ^{1}H and ^{31}P NMR and tandem mass spectrometry. From these analyses, the GGPL-III was found to be composed of 2 M fatty acid (C:16 and C:18), 1 M diacylglycerol, α-glucose, phosphocholine and amino residue. The glucose was found to be attached to the 3 position of diacylglycerol through α 1-3 linkage. More detailed chemical structure will be presented.

1. Matsuda K, Kasama T, Ishizuka I, Handa S, Yamamoto N, Taki T (1994) *J Biol Chem* **269**: 33123–28.

S6

Structure of a novel Heptose-Containing Eubacterial S-Layer Glycoprotein Glycan

T. Wugeditsch[1], P. Kosma[2], R. Christian[3] and *P. Messner*[1]
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[2] *Institut für Chemie, Universität für Bodenkultur, A-1180 Wien, Austria.*
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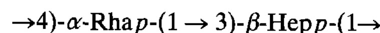
The glycan structure of the S-layer glycoprotein of '*Bacillus brevis*' ATCC 12990 was analysed. Currently, the taxonomic affiliation of this organism is being reinvestigated. In contrast to many other S-layers of eubacteria the S-layer of this strain was readily soluble in buffer solutions. Therefore a new strategy for isolation and purification of the S-layer glycoprotein and Pronase-derived glycopeptides including buffer extraction, FPLC, gel filtration, ion exchange chromatography, and chromatofocusing had to be developed.

Based on one- and two-dimensional NMR measurements the high-molecular-weight glycans of several isolated S-layer glycopeptides were identical and consisted of disaccharide repeating units with the constituents rhamnose and glycero-mannoheptose with the sequence:



$$\rightarrow 4)\text{-}\alpha\text{-Rha}p\text{-}(1 \rightarrow 3)\text{-}\beta\text{-Hep}p\text{-}(1\rightarrow$$

Supported by the Austrian Science Foundation, project S7201-MOB and the Austrian Ministry of Science, Research, and Arts.

1. Messner P, Sleytr UB (1992) *Adv Microb Physiol* **33:** 213–75.

S6

Novel Sulfated Trisaccharides Produced from Fucoidan by a Novel Bacterial Endo-fucoidan-lyase Degradation and Elucidation of Long Side Chain of Fucoidan

T. Sakai[1], H. Kimura[1], K. Kojima[1], Y. Nakanishi[1], K. Ikai[2], and I. Kato[1]
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An electrophoretically homogeneous fucoidan was purified

from a brown seaweed, *Kjellmaniella crassifolia Miyabe*. We found that a kind of *Flavobacterium* secreted a novel fucoidan-degrading enzyme that digested about 20% of the fucoidan and produced novel sulfated trisaccharides. Each of the oligosaccharides was isolated respectively by anion exchange chromatography, and characterized by component sugar analysis, mass spectrometry, and 500 MHz 1D and 2D ^{1}H NMR spectrometry. The structures of the products were L-Fuc(3-*O*-sulfate)α1-3($\Delta_{4,5}$GlcAβ1-2)D-Man and L-Fuc(2,4-*O*-disulfate) α1-3($\Delta_{4,5}$GlcAβ1-2)D-Man(6-*O*-sulfate) and so on. The enzyme also produced hexa- or longer saccharides, such as L-Fuc (3-*O*-sulfate) α 1-3 ($\Delta_{4,5}$GlcAβ 1-2) D-Manα1-4GlcAβ1-2 (L-Fuc(3-*O*-sulfate)α1-3) D-Man early in the reaction. From the results, the existence of long side chains in the fucoidan was considered because all products contained unsaturated glucuronic acid, that is a trace of enzymatic digestion. To elucidate the existence, the fucoidan was hydrolysed in 0.5 M oxalic acid at 100 °C for 3 h before and after the enzymatic degradation. A polymer of (-4-D-GlcAβ1-2-D-Manα1-)$_{about\ 40}$ remained after hydrolysis of fucoidan before enzymatic degradation only. From these results, at least a chain of (-4-D-GlcAβ1-2-D-Manα1-)$_{about\ 40}$ that C-2 of mannosyl residue substituted by 3-*O*-sulfated L-fucose or 2,4-*O*-disulfated L-fucose, should exist in one molecule of the fucoidan. The novel endo-fucoidan-lyase cleaved D-Manα1-4 GlcA in the fucoidan eliminatively and produced the novel sulfated trisaccharides.

MONDAY 21 AUGUST, MORNING

PLENARY LECTURES

S7. 8.00am

Carbohydrate Interactions with Ca^{2+}-Dependent Animal Lectins

K. Drickamer
Glycobiology Institute, Department of Biochemistry, University of Oxford, Oxford OX1 3QU, UK.

Calcium-dependent (C-type) animal lectins target biological functions based on the locations and structures of their saccharide ligands [1]. Lectins in this group include receptors that recognize endogenous ligands, such as the selectin cell adhesion molecules and the asialoglycoprotein receptor, which mediates endocytosis of serum glycoproteins. Other C-type lectins such as serum mannose-binding protein mediate an innate immune response to pathogens. All of the C-type lectins contain structurally related carbohydrate-recognition domains (CRDs). Analysis of the CRD from a rat mannose-binding protein by X-ray crystallography reveals that the role of the conserved residues found in all C-type CRDs is to create the CRD fold, which forms binding sites for two calcium ions. The saccharide-binding site is in close proximity to one of the calcium ions. The 3 and 4 hydroxyl groups of mannose form a network of coordination bonds with the calcium and hydrogen bonds with glutamic acid and asparagine residues in the protein. Additional selectivity in sugar binding, such as preferential binding of certain CRDs to *N*-acetylgalactosamine over galactose, results from additional sugar-protein contacts that are being investigated by site-directed mutagenesis and physical methods. The higher order selectivity of intact C-type lectins for oligosaccharides reflects the presence of CRDs and their arrangement in a specific geometry in receptor oligomers. Current work suggests that many of the lectins contain groups of three CRDs held together by a common structural motif.

1. Drickamer K, Taylor ME (1993) *Annu Rev Cell Biol* **9**: 237–264.

S8. 8.30am

The Yeast Oligosaccharyltransferase Complex: Analysis of Mutants in the *OST1*, *OST2* and *OST3* Genes

R. Gilmore, S. Silberstein, D. Karaoglu, P. Collins, P. Rapiejko and D. Kelleher
Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School; Worcester, MA 01655, USA.

Oligosaccharyltransferase (OST) catalyses the transfer of high mannose oligosaccharide from a dolichol-linked donor (dolichol-P-P-GlcNAc$_2$Man$_9$Glc$_3$) to asparagine acceptor sites in polypeptides within the lumen of the rough endoplasmic reticulum. The OST from *Saccharomyces cerevisiae* has been purified as a protein complex composed of six subunits, two of which correspond to subunits of the yeast OST identified by genetic methods (Wbp1p and Swp1p). Yeast genes (*OST1*, *OST2* and *OST3*) that encode three of the subunits of the OST complex have now been isolated and sequenced. The protein encoded by the *OST1* gene is 28% identical to ribophorin I, a subunit of the mammalian oligosaccharyltransferase. Disruption of either the *OST1* gene or the *OST2* gene is lethal in haploid yeast. Temperature sensitive *ost1* or *ost2* mutants underglycosylate proteins *in vivo* and show reduced *in vitro* transfer of a high mannose oligosaccharide to an acceptor tripeptide. Growth of haploid yeast is not altered by disruption of the *OST3* gene. However, yeast lacking the *Ost3* protein show marked defects in glycosylation of a subset of proteins *in vivo*, and reduced *in vitro* oligosaccharyltransferase activity.

S9. 9.00am

Topology of Glycosphingolipid Metabolism, Function and Pathobiochemistry of Sphingolipid Activator Proteins

Konrad Sandhoff, Andreas Klein and Gerhild van Echten-Deckert
Institute for Organic Chemistry and Biochemistry, 53121 Bonn, Germany.

Glycosphingolipids form cell and differentiation specific patterns in the outer leaflet of the plasma bilayer membrane. The enzymes involved in their biosynthesis are compartmentalized in the ER and Golgi apparatus; their sites of residence and membrane topology will be discussed [1]. After endocytosis catabolism of membrane-bound gangliosides is catalysed by exohydrolases in the lysosomal compartment. Degradation of glycolipids with a short oligosaccharide moity needs the assistance of activator proteins [2]. Based on the analysis of atypical forms of lipid storage diseases with mutations in the activator proteins a new model for the topology of endocytosis and lysosomal digestion will be presented [2, 3]. Feeding of activators to cultured human fibroblasts being simultaneously deficient in several activators reveals the function of individual

activator proteins.

1. van Echten-Deckert G, Sandhoff K (1993) *J Biol Chem* **268:** 5341–44.
2. Fürst W, Sandhoff K (1992) *Biochim Biophys Acta* **1126:** 1–16.
3. Sandhoff K, Klein A (1994) *FEBS Lett* **346:** 103–7.

S7. CARBOHYDRATE-PROTEIN INTERACTIONS

Chairs: Donald Marcus, Nathan Sharon

S7. 9.50am

Site directed mutagenesis of contact amino acids in the combining site of a legume lectin

R. Adar and *N. Sharon*
Department of Membrane Research and Biophysics, Weizmann Institute of Science, Rehovot 76100, Israel.

Lectins of leguminous seeds comprise a large family of structurally homologous proteins with distinct specificities, even though their combining sites are in part superimposable, with an invariant constellation of three amino acids, two hydrophilic (Asn, Asp) and one hydrophobic (Phe, Tyr or Trp). The elucidation of the 3D structure of the Gal-specific Erythrina corallodendron lectin (ECorL) in complex with lactose [1] and the cloning and expression of the lectin in E. coli [2] permitted further examination of the factors determining the specificity of the lectin by site-directed mutagenesis. ECorL possesses a cavity at the combining site surrounded by Tyr^{108}, Pro^{134} and Trp^{135}, that can accommodate bulky substituents at C-2, such as dansyl [3]. Our results show that Trp^{135} contributes to the strong binding of GalNDns to the lectin, whereas Tyr^{108} does not. Contrary to predictions based on the 3D structure of the complex, Gln^{219} is not required for ligand binding; it however contributes to the high affinity of the lectin for LacNAc by interacting with the GlcNAc moiety of the disaccharide. The inability of ECorL to bind Glc or Man is not due to interference by the side chain of Ala^{218} in the variable part of the combining site, since mutation of this residue to glycine did not affect the specificity of the lectin. As expected, mutation to Ala of Asn^{89} and Asp^{133} that form key hydrogen bonds with bound Gal and of Phe^{131}, which interacts with it hydrophobically, resulted in compete loss of activity. Conclusions based on other mutants as well as double mutants will be reported.

Supported by a grant from the Basic Research Found of the Israel Academy.

1. Shaanan *et al.* (1991) *Science* **254:** 862.
2. Arango *et al.* (1992) *Eur J Biochem* **205:** 575.
3. Arango *et al.* (1993) *FEBS Lett* **330:** 133.

S7. 10.15am

Crystal Structure of the Plant Lectin Amaranthin and its Complex with the T-Disaccharide

Thomas R. Transue, Alexander K. Smith, Hanqing Mo, Irwin J. Goldstein and Mark A. Saper
Biophysics Research Division and Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109-1055, USA.

The crystal structure of the plant lectin *Amaranthus caudatus* agglutinin (ACA) has been solved in the unbound state and in complex with a disaccharide ligand. Each subunit of the 66 kDa homodimer contains two homologous beta-trefoil type domains connected by a short alpha helix. Other members of the beta-trefoil structural family include fibroblast growth factor, interleukin 1, hisactophilin, Kunitz-type trypsin inhibitors from *Erythrina* and soybean, and the lectin (B) chain from the cytoxic heterodimer ricin. While each of these proteins contains the same tertiary fold, similarity between their primary sequences is very weak.

The carbohydrate binding site of ACA is at the dimer interface, but on an exposed surface of the protein rather than in a cleft. No significant conformational changes of the protein are observed upon carbohydrate binding. The observed specificity and relatively tight binding (K_D about 1.6 μM) of Gal β1,3 GalNAc α-*O*-benzyl results from contacts with both sugars of this derivative of the T-disaccharide carcinoma antigen. In contrast ricin, the only other lectin known to have a beta-trefoil fold, binds lactose three orders of magnitude weaker and interacts with only the terminal galactose. Furthermore, while ricin's binding requires an aromatic side chain which interacts with the hydrophobic surface of galactose, ACA makes no such contact. Thus, while ACA is structurally homologous to the lectin chain of ricin, its mode of carbohydrate binding is different and more specific.

S7. 10.30am

Do Cytokines Recognize Glycans of GPI-anchor?

Katsuko Yamashita, Keiko Fukushima and Takashi Ohkura
Department of Biochemistry, Sasaki Institute, Kanda-Surugadai, Tokyo, 101, Japan.

Tumour necrosis factor-α, lymphotoxin and interleukin-1β have similar lectin-like characters. The precise carbohydrate binding specificities were investigated using inhibition assay by oligosaccharides to binding between cytokines and glycoproteins. These cytokines specifically bound human placental alkaline phosphatase, carcinoembryonic antigen, Trypanosoma variant surface glycoproteins and uromodulin released by phospholipase C, and ovalbumin, on the other hand, they did not interact with human transferrin, human orosomucoid, porcine thyroglobulin, and bovine ribonuclease B. The interaction was not inhibited by N-linked high mannose type and complex type oligosaccharides, mannitol-6-phosphate, ethanolamine-phosphate, inositol-1-phosphate and mannose-1-phosphate, but inhibited by mannose-6-phosphate, Manα1 $\rightarrow$ 6Manβ1 $\rightarrow$ 4GlcNAcβ1 $\rightarrow$ 4(Fucα1 $\rightarrow$ 6)GlcNac and $\pm$Galβ1 $\rightarrow$ 4GlcNAcβ1 $\rightarrow$ 4(GlcNAcβ1 $\rightarrow$ 2)Manα1 $\rightarrow$ 3(GlcNAcβ1 $\rightarrow$ 4){Manα1 $\rightarrow$ 6(Manα1 $\rightarrow$ 3)Manα1 $\rightarrow$ 6}Manβ1 $\rightarrow$ 4GlcNAcβ1 $\rightarrow$ 4GlcNAc. These results indicated that the specific C-6 substituted mannosyl residues are essential for interaction between

these cytokines and glycoproteins, and the added negative charged residues increase the binding strength. So, it is considered that these cytokines recognize the glycan portions of GPI-anchored glycoproteins on the plasma membranes.

S7. 10.45am

Lectin-mediated Gene Transfer into Airway Epithelial Cells

W. Yin and *P.-W. Cheng*
Department of Pediatrics, University of North Carolina, Chapel Hill, NC 27599, USA.

The purpose of this study was to examine whether the lectins which recognize cell surface carbohydrates could be used as gene transfer vectors. Lectins which bind to airway epithelial cells (CFT1) were identified by exposing the cultured cells which had been fixed in 4% paraformaldehyde-0.25% glutaraldehyde (4 °C, 10 min) to fluorescein-conjugated lectins with various carbohydrate specificities: Con A (Man), WGA (sialic acid/GlcNAc), SNA (sialic acidα2,6Gal), MAA (sialic acidα2,3Gal), UEA-1 (α1,2Fuc), GS-I (αGal/GalNAc), GS-II (GlcNAc), BPA (GalNAc), SBA (blood group $A_1 > A_2 > B$), PNA (Galβ1,3GalNAc), MPA (internal GalNAc), and DBA (αGalNAc). The four lectins, Con A, SNA, WGA, and MAA, which bound appreciably were conjugated with polylysine (PL) or histone (His) via disulfide linkages. The molecular conjugates (5.3 $\mu g\,ml^{-1}$) were used to deliver the pCMVlacZ plasmid (4 $\mu g\,ml^{-1}$) to cultured CFT1 cells. Relative to those (= 1.0) of the control cells, which were transfected with lectin + polycation + DNA, the β-galactosidase activities expressed in the cells transfected with lectin-PL (or His)-DNA complexes were: Con A-His, 7.7; SNA-His, 3.5; Con A-PL, 2.4; WGA-PL, 1.3; others, 1.0. In addition, Con A-His mediated gene transfer was dose dependent between 2.5 and 10 $\mu g\,ml^{-1}$. Maximal β-galactosidase activity of 41 was obtained at 10 $\mu g\,ml^{-1}$ of Con A-His. At higher concentration, the conjugate caused cytotoxicity. We conclude that lectin-polycation conjugates can deliver a reporter gene to airway epithelial cells in which the transfected gene can express.

Supported by Cystic Fibrosis Foundation.

S7. 11.00am

Maltose-Sulfate Oligosaccharides Inhibit Tube Formation (In Vitro Angiogenesis) by Microvascular Endothelial Cells

C. Foxall, Z. Wei, C. Peto, P. Fugedi and *B. K. Brandley*
Glycomed Inc., Alameda, CA, USA.

The behaviour of microvascular endothelial cells (EC) is central to many normal and pathological processes, including angiogenesis in vascular development, wound healing, inflammation and cancer. Some aspects of angiogenesis can be approximated using an *in vitro* tube-forming assay on basement membrane components. We selected a variety of small, sulfated oligosaccharides based on their ability to bind to bFGF (FGF-2), and tested them as modulators of rat epidydimal fat pad microvessel EC (REEC) tube formation on Matrigel. Tube formation began within 2 h after plating and reaching a peak at 10–12 h. Structure-activity relationship data generated using a series of malto-oligosaccharide polysulfates ranging from α-methyl-glucopyranoside tetrasulfate, to maltoheptaose with approximately 22 sulfates, indicated that sulfated structures as small as tetrasaccharides were capable of blocking REEC tube formation. Similar sulfated oligosaccharides with the reducing end glucose residue reduced also blocked tube formation, although longer structures were required for inhibition. The ability to inhibit tube formation correlated with bFGF binding, suggesting that these compounds may block angiogenesis by inhibition of bFGF function.

S7. 11.15am

Detection of a Non-covalent Oligosaccharide-protein Complex by Mass Spectrometry: Molecular Recognition of a *Salmonella* Hexadecasaccharide by a Single Chain Antibody

Jasna Peter-Katalinic[1], Brian N. Green[2], Alison E. Ashcroft[2], Saran A. Narang[3], C. Roger MacKenzie[3], N. Martin Young[3], Eleonora Altman[3] and David R. Bundle[4]
[1]*Institute for Physiological Chemistry, University of Bonn, D-53115 Bonn, Germany.*
[2]*FISONS/Organic Analysis, Biotech MS, Altrincham, Cheshire WA14 5RZ, UK.*
[3]*Institute for Biological Sciences, NRC, Ottawa, ON Canada K1A 0R6.*
[4]*Department of Chemistry, University of Alberta, Edmonton, AB Canada T6G 2G2.*

Electrospray mass spectrometry has been applied to study the binding of a hexadecasaccharide comprising four repeating units of a *Salmonella* lipopolysaccharide O-antigen by a single chain antibody Fv protein. The clustering process and the stoichiometry of the carbohydrate/protein complex was detected directly from the shift of the multicharged protein molecular ions in (+)ES-mass spectra. The protein complex is dependent on sterochemistry of the 3,6-dideoxyhexose moieties at the carbohydrate epitope.

S7. 11.30am

Glycosaminoglycan-binding Specificities of Annexin V

H. Utsumi, K. Kojima, H. Ogawa and I. Matsumoto
Department of Chemistry, Faculty of Science, Ochanomizu University, Tokyo, Japan.

The annexins are a family of Ca^{2+}-dependent phospholipid-binding proteins. We have previously demonstrated that bovine annexin IV binds to glycosaminoglycans and certain sialoglycoproteins in the presence of Ca^{2+} [1, 2]. This finding suggested that not only annexin IV but also other annexins are new types of lectins and function *in vivo* by binding to carbohydrates.

Annexin V has been identified as a placental anticoagulant protein and most recently was shown to be a paracrine-type neurotrophic factor for neocortical neurons [3]. In this study, we isolated annexin V from bovine brain extract and examined the glycosaminoglycan-binding specificities by the use of various glycosaminoglycan columns, which were prepared by coupling the carboxyl groups of glycosaminoglycans to amino groups introduced into the agarose gels via amide linkages.

The binding specificities revealed for annexin V were significantly different from those of annexin IV. Annexin V bound to heparin and heparan sulfate in the presence of Ca^{2+} but not to N-desulfated heparin and N, O-desulfated heparin. Annexin V did not bind to chondroitin sulfate A, B and C, and hyaluronic acid. These results suggest that annexin V interacts with cell surface heparan sulfate proteoglycans *in vivo*.

1. Kojima K *et al.* (1992) *J Chromatogr* **597:** 323–30.
2. Kojima K *et al.* (1992) *J Biol Chem* **267:** 20536–39.
3. Takei N *et al.* (1994) *Neurosci Lett* **171:** 59–62.

S7. 11.45am

High affinity antibodies against 3-FL (Le^x^, CD15) from a phage display library

D. M. Marcus, N. Weng and Q. Dinh
Baylor College of Medicine, Houston, TX 77030, USA.

We previously sequenced 8 anti-3-FL mAbs, obtained from four fusions, and found that they were all encoded by VH441, of the X24 family, and VK24B, and all mAbs had low avidity. We wanted to obtain higher avidity Abs and to ascertain if other light (L) chains could encode these Abs. We constructed a phage display library, in a modified pComb 8 vector, that contained random L chains and Fd segments enriched in VH domains of the X24 family. We selected phage with a 3-FL-BSA antigen, and obtained two clones, 23 and 24, whose avidity for this antigen was at least 100x higher than our hybridoma mAbs. Both Abs also bound sialyl Le^x^ very strongly, and 23 also bound nLc4Cer well. Sequencing revealed that the VH domains of both mAbs were encoded by VH441. The L chain of clone 24 was encoded by a gene from the VK Ox-1 family, and clone 23 by VK 9, and both L chains had shorter CDR1 segments than VK24B. These data indicate that the L chains of anti-3-FL Abs can be encoded by genes other than VK24B. The restriction in gene usage noted previously was probably regulatory in origin, rather than structural, because 3-FL is an autoantigen. To evaluate the contributions of L and H chains to the specificity and affinity of antigen binding, we have recombined the Fd segments of clones 23 and 24 with L chains from other anti-3-FL mAbs and anti-ganglioside Abs. Some of the new combinations did not bind 3-FL, and others bound very weakly and also exhibited altered specificity. Anti-carbohydrate Abs may exhibit less tolerance for swapping L chains than Abs directed against peptides.

Supported by research grant Al 17712 from the USPHS.

S7 POSTERS

S7

Multiple V_H and V_L Gene Segments Are Used by Murine Monoclonal Antibodies against Disialogangliosides

E. Cerato[1], S. Birkle[1], J. Portoukalian[2] and *J. Aubry*[1]
[1]*INSERM U.211, Faculté de Médecine-Pharmacie, 44035 Nantes, France.*
[2]*INSERM U.218, Centre Léon Bérard, Lyon, France.*

The gangliosides are poorly immunogens. A limited number of monoclonal antibodies (mAb) obtained from different fusions has been raised against their carbohydrate structure either in Balb/c or A/J mice. The sequences of variable (V) regions of the light (L) and heavy (H) chains of ten mAbs against disialogangliosides GD_2 and GD_3 were determined by a reverse transcription-polymerase chain reaction procedure. The V_H genes of the J558, 3609, 7183 families were expressed through multiple rearrangements with various D_H and J_H genes. The heavy chain CDR3 segments vary in length from 7–11 amino-acids. Interestingly two unique mAbs 8B6 and 7H2 respectively specific to GD_2-*O*-acetylated and GD_3-*O*-acetylated were encoded by the V_H genes of S107 and 7183 families. The V_H chains were paired with different Vk light chains derived from germ line genes. The comparison of mAbs 4G2 and 4G12 shows that their fine specificity for the disialogangliosides can be modulated by the change of L chain associated to the same H chain. In contrast to the restricted usage of V_H and V_L genes by mAbs against 3-fucosyllactosamine, multiple V_H and V_L gene segments of mAbs against disialogangliosides were used similarly to mAbs against other carbohydrate antigens such as α(1-6)dextran and group A streptococcal carbohydrate.

Supported by ARC, LNFLC and MRES.

S7

New Type of Carbohydrate-Carbohydrate Interaction

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[1]*Shemyakin Institute of Bioorganic Chemistry, Moscow, Russia.*
[2]*Research Institute of Children Hematology, Moscow, Russia.*

Our previous studies [1] has shown that zymosan (polysaccharide complex from yeast) interacted specifically with Man-PAA [2] (water soluble conjugate of α-mannose with polyacrylamide). In the present investigation we tested the possibility that this interaction has a carbohydrate-carbohydrate nature. As zymosan contains some protein in addition to the major components of mannan and glucan, it was necessary to primarily exclude the carbohydrate-lectin type of interaction. Rigorous zymosan treatment by pronase, trypsin or alkali did not abolish the affinity to Man-PAA-biot. At the same time periodate oxidation (48 h) gradually decreased zymosan reactivity to practically zero level. Individual polysaccharides from zymosan (Sigma) also preserved the ability to specific binding with a probe, Man-PAA-Flu. Binding of zymosan with Man-PAA-biot [2] and Man-PAA-Flu was dose- and calcium-dependent and it was inhibited by monosaccharides D-Man, L-Fuc, D-GlcNAc. The ability of zymosan to bind Sug-PAA-biot decreased in the series Man > Fuc > GlcNAc. The conjugates having higher Man contents more intensely bound with zymosan. The formation of zymosan complex with Man-PAA-Flu was reversible; the treatment of this complex with Man or EDTA completely released the probe.

The data obtained confirm the suggestion that the studied interaction is of a carbohydrate-carbohydrate type with the participation of zymosan polysaccharides. It is possible that in natural conditions this mechanism mediates the bacteria and yeast interaction with mannose-rich glycoproteins. Zymosan interaction with carbohydrate N-chains from glycoproteins has also been studied.

1. Mikhalchik EV, Korkina LG, Shiyan SD, Bovin NV (1994) *Biol Membrane* **11:** 581–87.
2. Bovin NV *et al.* (1993) *Glycoconjugate J* **10:** 142–51.

S7

Lectin-Carbohydrate Interactions – Is the *Erythrina corallodendron* Lectin Preference for *N*-Acetyllactose amine Over Lactose due to a Specific Recognition of the N-Acetyl Moeity?

T. Ekberg[1], Kai Ding[2], H. Eriksson[2], U. Lindström[1], H. O. Sjögren[2] and G. Magnusson[1]
[1] *Department of Organic Chemistry 2, Chemical Center, The Lund Institute of Technology, University of Lund, P.O. Box 124, S-221 00 Lund, Sweden.*
[2] *Department of Tumor Immunology, The Wallenberg Laboratory, University of Lund, P.O. Box 7031, S-220 07 Lund, Sweden.*

The 'galactose-binding' lectin from *Erythrina Corallodendron* (Ecor-L) has a 16-fold higher affinity for *N*-Acetyl lactoseamine over lactose, a difference in affinity corresponding to $\Delta\Delta G \approx 1.6$ Kcal mol^{-1}. The Ecor-L affinity for TMS-ethyl lactoside (containing a lipophilic $(CH_3)_3SiCH_2CH_2$-group in the anomeric position) was virtually same as for lactose. This was true also for 2,3-deoxygenated methyl lactoside and 1-deoxy lactose, whereas 3-deoxygenated TMS-ethyl lactoside showed a two-fold increased affinity for Ecor-L compared to lactose. Thus, complex formation seems to be favoured by the presence of a hydrophobic patch close to a polar group at Glu-2.

Is the glutamine residue that is located close to lactose Glu-2 and 3 (according to X-ray crystallography [1] involved in hydrogen bonding to the N-Ac group in *N*-Acetyl lactoseamine?

1. Shanaan B, Sharon N (1991) *Science* **254:** 862–866.

S7

Neoglycopolymer Ligand for Mannose-Binding Proteins: Transglycosylation with Endo-*β*-*N*-acetylglucosaminidase from *Arthrobacter protophormiae*

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We have reported that transglycosylation activity of endo-*β*-*N*-acetylglucosaminidase from *Arthrobacter protophormiae* can be enhanced by inclusion of organic solvent in the reaction mixture [1]. This finding was extended to synthesize intermediates useful for preparation of neoglycoconjugates. When 0.2 M GlcNAc-*O*-$(CH_2)_6NH_2$, GlcNAc-*O*-$CH_2CH{=}CH_2$, GlcNAc-*O*-$(CH_2)_3CH{=}CH_2$, GlcNAc-*O*-$(CH_2)_3NHCOCH{=}CH_2$, GlcNAc-*S*-$CH_2CONHCH_2CH(OMe)_2$ or GlcNAc-*S*-CH_2CN were used as acceptors in media containing 30% acetone, the transglycosylation yields were ca. 80%. The products are useful for modification of proteins or the construction of neoglycopolymers. A μmol-scale synthesis of $Man_9GlcNAc_2$-*O*-$(CH_2)_3NHCOCH{=}CH_2$ was accomplished in 90% yield, and the product was co-polymerized with acrylamide. The ratio of sugar side chain to acrylamide in this glycopolymer was 1:44 and the molecular weight was between 1 500 000 to 2 000 000 (by high performance gel filtration chromatography). The neoglycopolymer showed more efficient inhibitory activity for binding by recombinant rat mannose binding protein-carbohydrate recognition domains from serum and liver than soybean agglutinin.

1. J-Q Fan *et al.* (1994) *Glycobiology*, **4**, 735.

S7

Maltose-Sulfate Oligosaccharides have Differing Effects on the Growth of Endothelial Cells Isolated from Different Organs and/or Species

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The behaviour of microvascular endothelial cells (EC) is central to many normal and pathological processes, including angiogenesis in vascular development, wound healing, inflammation and cancer. We selected a variety of small, sulfated oligosaccharides based on their ability to bind to bFGF (FGF-2), and tested them as modulators of EC growth. Structure-activity relationship data generated using a series of malto-oligosaccharide polysulfates ranging from α-methyl-glucopyranoside tetrasulfate, to maltoheptaose with approximately 22 sulfates, indicated that, for human microvascular lung EC, sulfated malto-oligosaccharides inhibited cell proliferation. Increasing size correlated with increased growth inhibition, and growth inhibition correlated with their ability to bind to bFGF. The same compounds tested on rat epidydimal fat pad microvascular EC, however, showed a size-dependent stimulation of growth. The growth stimulation increased from no stimulation with α-methyl-glucopyranoside, to peak stimulation with 100 μg ml^{-1} maltotriose polysulfate, then decreased to no stimulation with maltoheptaose polysulfate. Experiments are continuing to determine if the differences seen are due to use of cells from different species, cells isolated from different organs, or differences in the culture media used.

S7

Use of Capillary Zone Electrophoresis for the Study of Low Affinity Interactions Between Heparin and Proteins

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A procedure is outlined for studying low affinity interactions between proteins and sulfated glycosaminoglycans (GAGs). It is based on the difference in electrophoretic mobility of a free protein as compared to the corresponding protein-GAG complex. The migration time of the protein in capillary zone electrophoresis (CZE) is determined in a series of experiments in which the concentration of ligand (heparin in our study) in the electrolyte solution is varied. With the recorded differences in mobility, binding curves can be constructed, and the binding constant determined. The method was applied to the interaction between antithrombin (AT) and low affinity heparin. This interaction is about a thousand times weaker (K_D = 19 μM) [1] than the interaction mediated by the specific pentasaccharide sequence present in high affinity heparin that mediates the strong interaction with AT (K_D = 19 nM) [1]. When the concentration of low affinity heparin in the system was varied from 0 to 1 mM, the mobility of antithrombin increased gradually. Graphical presentation of the data gave a binding curve aligned

with the theoretical curve for monovalent binding. Our estimation of the binding constant was 16 μM.

1. Nordenman B, Björk I (1978) *Biochemistry* **17:** 3339.

S7

Natural Human and Rabbit Anti-Carbohydrate Antibodies: Difference in their Interaction with Lactosylceramide and Lactosyl-Bovine Serum Albumin Conjugates

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In the course of our studies concerning active and passive immunization of cancer patients, we aimed at the synthesis of sugar-containing tumour associated antigens. To this end, we applied the maleinimido coupling method of sugar to protein bovine serum albumin (BSA). With the aid of this method we coupled lactose, sialyllactose, disialyllacto-*N*-tetraose and dimer Le^x sugar epitopes to BSA. The number of the coupled lactose sugar epitopes was e.g.: 1, 4, 6, 8, 10, 14, 19 and 22 per mol albumin. The characterization of these glycoconjugates was done with the aid of SDS-PAGE, chemical determination of sugar and protein contents, molecular exclusion HPLC and MALDI/MS. The interaction of human and rabbit immunoglobulins with lactosylceramide (Galβ1 $\rightarrow$ Glcβ1 $\rightarrow$ Cer) and Lac-BSA glycoconjugates was investigated with the aid of Elisa tests. The control native antigens: GM1 ganglioside, asialo-GM1 glycolipid, glycosphingolipid with Galα1 $\rightarrow$ 3Galβ- terminal sugar epitope, T- and Tn-glycoprotein were included in these experiments.

The presence of natural, low affinity anti-lactosyl antibodies in the plasma of healthy volunteers was determined. The amount of them was independent of the level of known natural anti-T, anti-Tn, anti-asialo-GM1 and anti-Galα1 $\rightarrow$ 3Galβ antibodies.

S7

Thermodynamics and Specificity of Oligosaccharide–Protein Interactions

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Many proteins implicated in disease states express their unique functions by recognizing and interacting with oligosaccharides. An understanding of the specificity of biomolecular interactions, and ultimately the design of specific inhibitors, requires a detailed characterization of the dynamic structures of proteins, ligands, and their complexes. NMR is a powerful tool with which to study lectin–carbohydrate interactions. Transferred nOe (trNOE) experiments can be used to determine the conformation of a ligand in the bound state. The incorporation of NMR active nuclei into the ligand allows only ^{1}H spins present in the oligosaccharide to be selectively detected.

The chemoenzymatic synthesis of ^{13}C labelled sialic acid (Neu5Ac), N-acetyl glucosamine (GlcNAc), and N-acetyl lactosamine (LacNAc: Galβ1-4GlcNAc) has been achieved on the milligram scale. GlcNAc was synthesized via a one-pot enzymatic synthesis of [U]-^{13}C glucosamine 6-phosphate utilizing [U]-^{13}C fructose or glucose as starting materials. ^{13}C-Neu5Ac was synthesized by base-epimerization of ^{13}C-GlcNAc to ^{13}C-N-acetyl mannosamine (ManNAc) and converted to ^{13}C-Neu5Ac by NeuAc aldolase in the presence of [U]-^{13}C sodium pyruvate. ^{13}C-LacNAc was synthesized in a one-pot reaction, coupling the production of UDP-[U]-^{13}C galactose with ^{13}C GlcNAc in the presence of lactose synthetase to produce ^{13}C labelled LacNAc.

Sialic acid and N-acetyl lactosamine are moieties commonly found in glycolipids and glycoproteins; the incorporation of these labelled sugars will allow the investigation of a large number of carbohydrate–protein systems by NMR. Initially both ^{13}C-LacNAc and ^{13}C-Neu5Ac will be coupled together to synthesize ^{13}C-α2,6-sialylactosamine (Neu5Acα2-6Galβ1-4GlcNAc) which binds the *Triticum Vulgaris* lectin. Preliminary studies using unlabelled ligand have shown that this complex is amenable to NMR investigation using 2D-transferred NOESY experiments.

S7

Syntheses of Deoxygenated Methyl α-Isomalto-oligosaccharides and their Binding with an Anti (1 $\rightarrow$ 6)-dextran Antibody 35.8.2H

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This laboratory is studying the binding of monoclonal, dextran-specific antibodies. In the past a systematic method to map the H-bonding interaction between saccharides and antibodies were developed [1]. We are now studying IgG 35.8.2H, an antibody capable of binding internal antigenic epitopes of the dextran [2]. We have found that in IgG 35.8.2H there is a perturbable tryptophanyl residue in the general combining area, but it is distant to the subsite possessing the highest affinity for its glucosyl residue. In order to probe possible H-bonding interactions we have prepared methyl α-isomalto-oligosaccharides specifically deoxygenated at different positions [3–6], and here report on some of our binding studies.

1. Glaudemans CPJ (1991) *Chem Rev* **91:** 25–33.
2. Matsuda T, Kabat EA (1989) *J Immunol* **142:** 863–70.
3. Petráková E, Kovác P, Glaudemans CPJ (1992) *Carbohydr Res* **233:** 101–12.
4. Petráková E, Glaudemans CPJ (1994) *Glycoconjugate J* **11:** 17–22.
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6. Petráková E, Glaudemans CPJ, unpublished data.

S7

Evidence for Two Active Sites in Rat Liver *N*-Acetylglucosaminide β1-4-Galactosyltransferase

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n-Octyl-β-glucoside (OG) was found to serve as acceptor for a β1-4-galactosyltransferase (β1-4-Gal-T) from rat liver Golgi. Another acceptor for β1-4-Gal-T was benzyl-β-glucosaminide

(BGN). The galactosyl-transferase product obtained from BGN was shown to be Galβ1-4BGN by permethylation and subsequent analysis by fast atom bombardment mass spectrometry (FAB MS).

Experiments using OG and BGN as substrates and mutual inhibitors resulted in mixed (non-competitive; $K_I = K_I^i$) inhibition of OG galactosylation by BGN and vice versa. The galactosylation of ovalbumin (OA), another substrate for β1-4-Gal-T, was also inhibited by BGN in a non-competitive way. These results suggested that take β1-4-Gal-T has two active sites, which influence each other.

In order to get a closer look at this phenomenon the 'divalent' acceptor 1, 10-0-bis-(*N*-acetylglucosaminyl)-decanediol (GN_2DEC; [1]) was synthesized and used as substrate for β1-4-Gal-T. GN_2DEC was converted to a mono- and a di-β1-4-galactosylated product (demonstrated by permethylation and FAB MS). With increasing acceptor concentration the relative amount of diGal-GN_2DEC decreased from 60% (at 50 μM GN_2DEC) to 13% (at 200 μM GN_2DEC). This fact could be explained by a displacement of one end of the 'divalent' acceptor from one of the active sites of β1-4-Gal-T by an increasing number of free GN_2DEC molecules. This result again supports the idea that β1-4-Gal-T has two active sites.

Our model is in agreement with the findings of Ats *et al.*, who suggested two separate GlcNAc binding-sites in bovine galactosyltransferase [1].

1. Ats S-C *et al.* (1994) *Carbohydr Res* **252**: 325–332.

S7

Conformation of an Oligosaccharide Accessed by Molecular Dynamics Simulations and Selected in the Lectin-Carbohydrate Complexes

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The overall shape of N-linked oligosaccharides has generally been considered to be dependent on the changes in the orientation of the α1,6-arm brought about merely by the two possible values of χ, $-60°$ and $180°$. Molecular dynamics (MD) simulations showed that the heptasaccharide Man-α1,6(Man-α1,3)(Xyl-β1,2)-Man-β1,4-GlcNAc-β1,4-(L-Fuc-α1,3)-GlcNAc, the carbohydrate moiety of *Erythrina corallondendron* lectin (EcorL) accesses a rare conformation that has been observed in the crystal structure of EcorL, but was not predicted from NMR. The MD simulations of this heptasaccharide and other N-linked oligosaccharides reveal that the orientation of the α1,6-arm is not only affected by changing χ but also by changing ϕ and ψ while keeping χ constant. The conformation ϕ_6, ψ_6, $\chi_6 = -62°$, $76°$, $-67°$, accessed rarely in MD simulations, that has been found in the crystal structure, might present a better complementary surface to bind to the symmetry-related lectin molecule in the crystal. If the heptasaccharide is not in this conformation while initiating the binding process the conformation around Man-α1,6-Man will be altered by changing ψ from its preferred value of 180° to 76°, to provide better complementary surface and to form additional hydrogen bonds. The conformational data derived from MD simulations not only provide a stereochemical explanation for the observed structures in the crystals of the lectin-oligosaccharide complexes, but also reveal a range of conformations an oligosaccharide can access, information which is vital for understanding carbohydrate-protein interactions.

S7

Isothermal Titration Calorimetry Characterization of Rat Mannose-binding Protein Carbohydrate-recognition Domains

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The carbohydrate-recognition domain (CRD) of the serum and liver mannose-binding proteins (MBP) have been purified from a bacterial expression system. Sensitive isothermal titration calorimeters (ITC) allowed direct measurement of the heats of binding for a panel of methyl pyranosides which are known to bind these lectins. The binding isotherms obtained from these data were analysed by least-squares fitting to determine association constant, molar enthalpy, free energy, and molar entropy of binding. Stoichiometry was determined by allowing the least-squares fitting programme to float the parameter n, the number of sites. For binding of mannose, the data fit $n = 2$ for the liver CRD, and $n = 1$ for the serum CRD. Consistent with this difference in stoichiometry for mannose, ITC measurements for binding a synthetic bivalent mannose-terminated cluster ligand show K_A in the range of 10^3 for serum MBP CRD and 10^5 for liver MBP CRD. The enhanced binding affinity for liver CRD known as the cluster effect is apparently enabled by an additional mannose-binding site in the liver CRD that is not observed in the homologous serum CRD.

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S7

Structural Requirements for Platelet-Derived Growth Factor-Heparin Interaction

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Platelet-derived growth factor (PDGF) plays an important role in the pathogenesis of atherosclerosis by inducing abnormal smooth muscle cell (SMC) proliferation in the arterial wall. Previous studies indicate that heparin and heparan sulfate bind PDGF and inhibit PDGF-induced arterial SMC growth *in vitro*. In this study we have analysed the structural requirements in heparin needed for binding to homodimers of long PDGF-A chains (PDGF-AA_{125}) by using a nitrocellulose filter-trapping assay of protein-heparin complexes formed in solution. Intact heparin and heparin fragments $\geqslant$18 disaccharide units in length showed saturable, high-affinity binding to PDGF-AA_{125}. The smallest fragment with PDGF-AA_{125} binding activity was an octamer showing about 30% binding compared to $\geqslant$18-mer fragments while 14-mer and larger fragments showed maximal binding. We next characterized the ability of differentially modified $\geqslant$18-mer heparin fragments to inhibit the heparin-PDGF-AA_{125} interaction. Only unmodified (fully sulfated) heparin was able to entirely inhibit the binding at concentrations of 0.5–5 $\mu g\,ml^{-1}$. Selectively *N*-desulfated and 2-*O*-desul-

fated heparin preparations with normal 6-*O*-sulfation inhibited the binding 48% and 45% at 5 $\mu g\,ml^{-1}$, respectively. De-*N*-sulfated and de-6-*O*-sulfated heparins carrying 35% or 70% of the normal 2-*O*-sulfate had no inhibitory effect. Interestingly, *N*-sulfated heparins with 35% or 70% 2-*O*-sulfate contents of normal heparin, but lacking 6-*O*-sulfate inhibited the binding only 27% and 34%, respectively. The finding that 6-*O*-desulfated but fully *N*-sulfated and up to 70% 2-*O*-sulfated heparin had relatively weak inhibitory effect may indicate that the 6-*O*-sulfate groups are particularly important for the PDGF-AA_{125}-heparin interaction.

S7

Ganglioside Induced Adherence of BTx and TTx to Adducin

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Preincubation of botulinum toxin serotype A (BTxA) or of tetanus toxin (TTx) with GT1b was found to enhance adherence of BTxA and be required for adherence of TTx to two proteins on blots of SDS-PAGE separated synaptosomal proteins. One protein, identified previously, was synapsin I. The second had an apparent molecular mass of ~116 kDa. Preincubation of either neurotoxin with GD3 resulted in greater adherence to this protein than was seen with GT1b. A purified preparation of the water soluble ~116 kDa protein was obtained by preparative column SDS-PAGE and 2D gel electrophoresis. Since the amino terminus of the protein was blocked, N-terminal amino acid sequence analysis was done using tryptic fragments isolated by HPLC. Data bank analysis of the N-terminal sequences obtained (IENPN QFVPL YTDPQ EVLDM R, and HKSEV EIPAT VTAFV FEEDG APVPA LR) indicated that the protein was adducin, a cytoskeletal protein. Based on these observations, we suggest that binding of the neurotoxin to gangliosides of the 'b' series present in nerve terminals induces a conformational change which permits either the intact or light chain portion of the toxin to adhere to synapsin and/or adducin. The resultant localization of the toxin would enhance the light chain catalysed proteolysis of a protein involved in the 'docking' of synaptic vesicles.

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S7

What Defines Specificity of Chemokine-Glycosaminoglycan Interaction?

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The large family of chemokines act on a broad range of different cells after binding to distinct receptors. Heparin/heparan sulfate chains have been postulated to assist in the process of individual chemokine action, participation ranging from passive sequestration/presentation of ligand to active promotion of the signalling process by direct modulation of receptor-ligand interaction. In order to analyse whether different chemokines exploit the structural variability of heparan sulfate sequences and have specific requirements for recognition of glycosaminoglycan chains, we have used both modified heparin fragments as well as heparan sulfate chains as tools. The strategy has been based on chemical modification of heparin, fragmentation of the chains and application of ^{3}H-labelled fragments to an in-solution trapping assay with the appropriate protein. The procedure enables selective enrichment of binding sequences which can be analysed on molecular level and tested for their biological effects. Several heparin binding chemokines show distinct motif-requirements to interact with glycosaminoglycan chains. The spectrum of 'specificity' in recognition ranges from high-sulfation blocks in the polysaccharide with otherwise no defined specific sequence requirements, as in the example of the cytokine interleukin-8, to a more defined requirement for specific sulfate groups at specific positions, as exemplified for the basic fibroblast growth factor. Our findings underline the wide possibilities of tissue specific modulation of chemokine action by heparan sulfate chains present on cell surfaces and in extracellular matrices.

S7

Recognition of Carbohydrates by the Macrophage Mannose Receptor

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The macrophage mannose receptor mediates binding and internalization of glycoconjugates terminating in mannose, fucose or *N*-acetylglucosamine. The receptor is believed to act as a molecular scavenger by clearing endogenous proteins which bear high mannose oligosaccharides, such as lysosomal enzymes, and by phagocytosing micro-organisms with a high density of mannose at their cell surfaces. The extracellular portion of the receptor consists of a cysteine-rich domain, a fibronectin type II repeat and eight segments related in sequence to Ca^{2+}-dependent carbohydrate-recognition domains (CRDs) of animal lectins. The carbohydrate-binding activity of the receptor resides in the CRDs. A single CRD, CRD 4, can mimic the monosaccharide-binding properties of the receptor, but binds poorly to glycoproteins. CRDs 4 and 5 form a ligand binding core sufficient to bind some ligands with high affinity but the affinity of the intact receptor for yeast mannan is only attained when CRDs 4–8 are present. In contrast to the mannose-binding proteins and asialoglycoprotein receptor, which appear to have a relatively rigid arrangement of CRDs determined by association of multiple subunits, the multiple CRDs in the mannose receptor may be arranged more flexibly to allow interaction with a more diverse set of oligosaccharides. Current studies are aimed at determining how each CRD interacts with sugars and how the CRDs are arranged to match the conformation of oligosaccharide ligands. NMR studies on CRD 4 produced in bacteria indicate that this CRD binds mannose, fucose and *N*-acetylglucosamine at the same site. The hypothesis that the spatial arrangement of the CRDs is important in determining affinity and specificity of binding is being tested using a series of mutant proteins in which the length of the spacer region between CRDs 4 and 5 has been changed. Binding of these mutants to carbohydrates is being studied.

S7

A New Sensitive Assay for the Evaluation of Interaction Between Basic Fibroblast Growth Factor and Heparin or Heparan Sulfate

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Basic fibroblast growth factor (bFGF, FGF-2) is a member of the heparin-binding growth factor family that interacts with cell surface and extracellular matrix heparan sulfate proteoglycans (HSPG). These interactions have significant biological functions, such as protection of the growth factor, mitogenic activity, and angiogenesis. Several assays used to study the binding of bFGF with HSPG have used cells as detectors of binding. These have included binding of transfected UC 729-6 cells to immobilized bFGF, proliferation of adrenal cortex endothelial (ACE) cells, and angiogenesis in the chorioallantoic membrane of fertilized chick eggs.

The assay we have developed eliminates the need to use cells in assessing bFGF and HSPG binding. This assay uses biotinylated heparan sulfate or heparin to bind to bFGF adsorbed to 96-well microtitre plates. Streptavidin conjugated with alkaline phosphatase is reacted with the labelled glycosaminoglycan. The amount of heparan sulfate or heparin bound is determined by the enzyme reaction with p-nitrophenylphosphate read as $O.D._{405}$.

This assay can be used for direct binding or as a competition assay. Potential inhibitors of bFGF and HSPG binding can be added with the labelled material and inhibition of binding measured by the reduction in signal. We have shown the heparin and heparan sulfate inhibit binding whereas chondroitin sulfate, dermatan sulfate, and keratan sulfate do not. Additionally we have shown that sulfated malto-oligosaccharides inhibit heparan sulfate binding to bFGF and that their potency increases with increased size. Thus this assay provides a convenient assay for the study of bFGF-glycosaminoglycan interactions as well as a rapid screen for potential bFGF inhibitors.

S7

Native and Asialo-Tamm-Horsfall Glycoproteins as Important Ligands for the Detection of GalNAcβ1→ and Galβ1 → 4GlcNAc Active Lectins

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The binding properties of human Tamm-Horsfall Sd(a+) urinary glycoprotein (THGP) and asialo-THGP with various applied lectins was investigated by quantitative precipitin and precipitin inhibition assays. Both glycoproteins completely precipitated *Abrus precatorius* agglutinin (APA). They also reacted well with *Wistaria floribunda* (WFA), *Glycine max* (soybean, SBA), and *Ricinus communis* agglutinins and precipitated over 78% of the lectin nitrogen added, but reacted poorly or weakly with all α-anomeric GalNAc specific lectins. The glycoprotein-lectin interaction was inhibited by GalNAcβ1→, Galβ1 → 4GlcNAc, or by both. The findings suggest that Sd(a+)THGP and asialo-THGP are among the best water-soluble glycoprotein ligands for GalNAcβ1→ and Gal β1 → 4GlcNAc active lectins.

S7

Surface Plasmon Resonance Analysis of Ligand Binding by Mutants of an Anti-Carbohydrate Antibody

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Mutant forms of a single-chain Fv (scFv) specific for the Salmonella serogroup B *O*-polysaccharide were isolated from phage libraries and analysed for their ligand binding kinetics by surface plasmon resonance. Affinities for BSA-*O*-polysaccharide conjugate were calculated by equilibrium binding analysis and from rate constants derived from linear and non-linear analysis of the binding data. It was found that the phage panning process selected for mutants which tended to form dimers, and that the presence of even trace amounts of dimer in the purified scFvs seriously compromised the calculation of association rate constants, due to avidity effects giving biphasic association kinetics. The affinities obtained for homogeneous monomer samples, with dissociation rates obtained in the presence of free trisaccharide ligand, were in good agreement with values derived from titration microcalorimetry. Dimeric samples showed monophasic association with 5–10 fold higher rates and biphasic dissociation with equal or 30 fold lower rates respectively than monomers. These results resolve some of the conflicts between surface plasmon resonance and microcalorimetry data, and quantify the effects of valence in this protein-carbohydrate interaction system.

S7

L5, A Functional Antigen in Early Neural Development is Le^x (SSEA-1)

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L5 is a murine antibody which has been shown to bind to neurons and astrocytes of the cerebellum. The L5 antigenic determinant is expressed on multiple glycoproteins: the neural cell-adhesion molecule L1, the Thy-1 glycoprotein, the chondroitin sulphate proteoglycan astrochondrin and several components not yet characterized. In early chick embryo, the L5 antigen is expressed initially by cells that are competent to respond to neural inducing signals but becomes rapidly restricted to and upregulated in the developing nervous system. L5 antibody interferes with neural induction *in vivo* suggesting that the L5 epitope itself plays a role during early neural development. The L5-positive glycoproteins isolated from murine and pig brains were shown to lose their L5 immunoreactivity after treatment with peptide *N*-glycosyl hydrolase F but there was no change after endoglycosidase D and H or chondroitinase ABC. N-linked oligosaccharides were released

by hydrazinolysis from L5-positive glycoproteins, converted into neoglycolipids, and L5-immunoreactive components were analysed by chromatogram binding assays and TLC-LSIMS [1]. In addition a panel of neoglycolipids of the blood group series were assessed for L5 immunoreactivities, and the free oligosaccharides were teated as inhibitors of L5 antibody binding. The L5 epitope was thus identified as Le^x (SSEA-1).

1. Feizi *et al.* (1994) *Methods Enzymol* **230:** 484–519.

S7

Effect of N-glycan on the Interaction of Fibronectin and its Receptor

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We have studied the role of N-glycan in the interaction between fibronectin (Fn) and fibronectin receptor (FnR). When the HT1080 cells are treated with 1 $\mu g\, ml^{-1}$ tunicamycin (TM), the synthesis of N-glycan is depressed dramatically. Fn deprived of N-glycan can still be secreted, and the amount of FN secreted is similar to the cells without TM treatment. Experimental binding of human plasma Fn to the FnR on the cell surface shows that the Fn binding sites on the cell surface decreased by 80% after TM treatment, the binding affinity (by Scatchard analyses) and the endocytosis rate of FnR in the TM treated cell do not alter. Since not all FnR molecules on the cell surface are deprived of N-glycan after TM treatment, Fn binding sites on the treated cell surface may represent those FnR molecules which still have N-glycan. Also FnR without N-glycan cannot be assembled on to the cell membrane. The same result has been obtained from the interaction between human placenta Fn and FnR. It was well known that N-glycan of plasma Fn is biantennary and placenta Fn is triantennary and tetraantennary. The binding affinities of these two kinds of Fn ligand for FnR are similar. This suggests that the structural difference of N-glycan of Fn does not affect Fn binding to FnR.

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S7

A Monoclonal Antibody Against Rat Liver Dipeptidyl Peptidase IV Binds to a Common Glycan Epitop on Glycoproteins and Glycosphingolipids

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The monoclonal antibody (mab) 25D (IgG3 isotype) was developed against the glycoprotein dipeptidyl peptidase IV (DPP IV) from rat liver plasma membrane. As previously described, several findings indicated the involvement of sialylated N-glycans in antigen antibody interaction [1]. To characterize the epitope we investigated the affinity of mab 25D to naturally occurring glycosphingolipids and artificial neoglycolipids derived from N-glycans of antigenic glycoproteins in antibody overlay assays on thin layer chromatograms (TLC).

Discrete glycolipid bands were immunostained by mab 25D on TLC of monosialogangliosides from granulocytes. Using the monoclonal antibodies HD66 with known specificity [2] as reference these bands could be identified as 2-6 sialosylneolactotetraosylceramid and 2-6 sialosylneolactohexaosylceramid. Thus mab 25D can be filed as a CD76 specific antibody.

Rat serum was found to contain a great number of glycoproteins which are immunostained by mab 25D. N-glycosidically bound oligosaccharides were cleaved from these glycoproteins by PNGase F, separated from peptides and coupled to L-1,2-dihexadecyl-sn-glycero-3-phosphoethanolamine (DHPE). The mixture of neoglycolipids was separated by tlc and immunostained using mab 25D. One single neoglycolipid band was stained, which was assigned by the relative mobility to a group of neoglycolipids derived from bisialylated N-glycans.

From the findings the epitope of mab 25D can be assigned to the tetrasaccharide structure NANA-α2-6-Gal-β1-4-GlcNac-β1-3-Gal-β1-, which is common to the above mentioned gangliosides and the terminal region of N-glycans with repeating N-acetyllactosaminyl groups localized in at least one of the antennae.

Since number and chain length of prolonged N-glycans in cell surface glycoproteins is often associated with the differenciation of the cell, mab 25D is expected to be a useful tool as a differentiation marker.

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2. Kniep B, Mühlradt PF, Dörken B, Moldenhauer G, Vilella R, Schwartz-Albiez R (1990) *FEBS Lett* **261:** 347–49.

S8. DOLICHOL-LINKED PATHWAYS REGULATING GLYCOSYLATION

Chairs: Phil Robbins, Markus Aebi

S8. 9.50am

Introduction by Phil Robbins

S8. 9.55am

N-linked Glycosylation of Proteins in the Endoplasmic Reticulum of *Saccharomyces cerevisiae*

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N-linked glycosylation of proteins is a highly conserved process in the endoplasmic reticulum (ER) of eukaryotic cells. The core oligosaccharide $Glc_3Man_9GlcNAc_2$ is transferred from the lipid dolichol phosphate to selected asparagine residues of nascent polypeptide chains, a reaction catalysed by the N-oligosaccharyl transferase complex.

Using yeast genetic techniques, we have identified different components of this multimeric enzyme. To study the complex biosynthetic pathway of the lipid-linked core oligosaccharide, we have designed novel approaches to identify yeast genes

involved in this process. We have isolated different loci encoding enzymes necessary for the synthesis of the core oligosaccharide. The cloning of these genes will make it possible to clarify the topology of oligosaccharide biosynthesis and its organization at the ER membrane.

S8. 10.25am

2-D NMR Analyses Reveal a Specific Interaction Between Polyisoprenols (PIs) and the Polyisoprenol Recognition Sequences (PRS) in Model Membranes

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NeuE and *KpsM*, two proteins implicated in the synthesis and/or translocation of the polysialic acid capsule in neuroinvasive *E. coli* K1, both contain a 13 amino acid polyisoprenol recognition sequence (PRS) in the transmembrane domain of the proteins.

1D and 2D (TOCSY) 500 MHz ^{1}H-NMR studies of the *NeuE* peptide showed significant changes in the chemical shifts of the NH protons and α protons on selective amino acid residues in the presence of the polyisoprenols (PIs), dolichol (C_{95}), C_{95}-phosphate (C_{95}-P) and undecaprenyl phosphate (C_{55}-P).

2D NOE and NH-CαH vicinal coupling constants indicated amino acid residues 1 to 7 in *NeuE* contained more α-helix-like character, while residues 8 to 13 contained a more extended β-sheet-like conformation. Based on these 2D NOE results, and on energy minimization and simulated annealing methods, we have obtained a 3D docking structure of the *NeuE* peptide binding to the PIs. These results show that Leu^1 and Leu^6 interact specifically with C_{95}, and that Ile^3, Leu^6 and Ile^7 make direct contact with C_{95}-P. Docking induces a conformational change in the *NeuE* peptide since a more compact structure in the β-sheet region of the peptide occurs after contact with PI. Further, *NeuE* has a slightly different conformation when bound to C_{95}, compared to C_{95}-P. This may result from a slightly different conformation and orientation of C_{95} and C_{95}-P in viscous medium.

An energy minimized structure of C_{95}-P and C_{55}-P was determined, which unexpectedly revealed an overall length of 33 Å and 20 Å, respectively. Molecular modelling showed that each bind several PRS peptides. This finding supports our earlier hypothesis of a bifunctional role for the PIs. The central idea is that these 'super-lipids' may function as a flexible matrix or scaffolding to organize and tether proteins of multienzyme complexes in order to link functions of biosynthesis and translocation.

S8. 10.40am

Phytanyl-linked *N,N'*-Diacetylchitobiose as an Alternative Substrate for Recombinant and Native Yeast Mannosyltransferases

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Dolichol is utilized *in vivo* as the anchor on which the precursor for N-linked oligosaccharides is assembled, but previous results have indicated that phytanol can substitute for dolichol as the substrate anchor for yeast β-1,4-mannosyltransferase and Dol-P-Man synthetase. Dolichyl- and phytanyl-linked *N,N'*-diacetylchitobiose were synthesized chemically and both were used successfully as substrates for a recombinant decahistidinyl-tagged transmembrane-domain-deleted form of the yeast β-1,4-mannosyltransferase (Alg1) which was expressed in *E. coli* and immobilized on a metal chelating resin. The β-[^{3}H]mannosylated lipids generated by this enzyme were then used as substrates for the subsequent mannosyltransferases from yeast or rat liver microsomes. After mild acid hydrolysis the products were analysed by Bio-Gel P4 and high performance anion exchange chromatographies. It was found that multi-nanomolar quantities of both dolichyl- and phytanyl-linked substrates were mannosylated to form $Man_5GlcNAc_2$. Digestion with *Aspergillus* mannosidase indicated that the correct 'anabolic' isomer of $Man_5GlcNAc_2$ had been formed with the phytanyl-linked substrate. Subsequently, at the level of 50 pmol of lipid-linked substrate, some further mannosylation to $Man_7GlcNAc_2$ and $Man_9GlcNAc_2$ was detected with both dolichyl- and phytanyl-linked substrates – the amount of Dol-P-Man generated endogenously was not limiting. Our results indicate: that a recombinant truncated form of yeast β-1,4-mannosyltransferase can be used to generate lipid-linked oligosaccharides with only one residue labelled; that removal of the transmembrane domain does not affect the ability of yeast β-1,4-mannosyltransferase to utilize the dolichyl-linked substrate; and that dolichol is not necessary *in vitro* as part of the substrate for the mannosyltransferases in the biosynthetic pathway for N-glycosylation.

S8. 10.55am

The Topography of Yeast Dolichol-Phospho-Mannose Synthase in Membranes

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Dolichyl-P-mannose synthase is a membrane-bound enzyme in the endoplasmic reticulum that catalyses the formation of a key intermediate for the biosynthesis of asparagine-linked oligosaccharides, O-linked mannosyl units, and the glycosyl-phosphatidylinositol anchor found on many membrane-bound proteins. Yeast synthase has been cloned [1], and the recombinant enzyme was purified to homogeneity from an *E. coli* high-expression vector. Little information is currently available, however, about the interaction of the protein with lipids or about the topology of the enzyme in ER membranes. We have demonstrated that Cys-93 is located near the substrate-binding site of the enzyme but is not required for catalysis. Detergent solubilized enzyme is also inhibited by diethyl pyrocarbonate (reversible by hydroxylamine) indicating that a reactive histidine residue is involved in the catalytic mechanism. In addition, the enzyme is inhibited by amine reactive reagents including 3,3'-Dithiobis(sulfosuccinimidylpropionate) and *N*-hydroxysuccinimidobiotin. Substrates protect against inactivation by all of these reagents, suggesting that the reactive amino acids are located in or near the active site of the enzyme. Reconstitution

of the enzyme with phospholipids protects against inactivation providing evidence that the active site of the enzyme lies in close proximity to the membrane.

Supported by NIH Grant GM47492.

1. Orlean P, Albright C, Robbins PW (1988) *J Biol Chem* **263**: 17499–507.

S8. 11.10am

Protein Phosphorylation: A 'Master Switch' Regulates Dolichol-Linked N-Glycosylation Pathway

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Increased glycosylation of eukaryotic asparagine-linked glycoproteins by β-adrenoreceptor stimulation was independent of intracellular Dol-P pool but dependent upon increased oligosaccharide-PP-Dol synthesis and turnover.

Biochemical studies supported the fact that it was not due to increased gene expression but perhaps due to a phosphorylation of Dol-P-Man synthase by a cAMP-dependent protein kinase (PK). This claim was confirmed by observing a two-fold increase in the V_{max} and detection of a 32 kDa phosphoprotein comigrating with the enzyme activity on SDS-PAGE, responding monospecifically to the antibody and coeluting with the synthase from an antibody-affinity column after protein phosphorylation. PK type I (PKI) deficient mutants which were synthesizing quantitatively less but full-length oligosaccharide-PP-Dol showed a three- to four-fold higher K_m for GDP-mannose and a reduced K_{cat}.

Regulation of Dol-P-Man synthase by PKI strongly supported its cytoplasmic orientation. In a series of immunocytochemical studies we have now observed that Dol-P is not only unidirectionally distributed but co-localized with the synthase.

S8. 11.25am

Endoplasmic Reticulum-to-cytosol Transport of Free Polymannose Oligosaccharides in Permeabilized HepG2 Cells

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Free polymannose oligosaccharides rapidly appear in both the vesicular and cytosolic compartments of HepG2 cells during glycoprotein biosynthesis. Here we investigated the possibility that some free oligosaccharides, originating in the lumen of the endoplasmic reticulum (ER), are transported directly into the cytosol. Incubation of metabolically radiolabelled cells, permeabilized with streptolysin O, in the absence of ATP at 37 °C led to the intravesicular accumulation of free $Man_9GlcNAc_2$ which was generated from dolichol-linked oligosaccharide in the ER. This oligosaccharide remained stable within the permeabilized cells unless ATP was added to the incubations at which time the $Man_9GlcNAc_2$ was partially converted to $Man_8GlcNAc_2$, and both these components were released from an intravesicular compartment into the incubation medium. In contrast when permeabilized cells, primed with either free triglucosyl-oligosaccharide or a glycotripeptide, were incubated with ATP both these structures lost a single mannose residue but remained associated with the permeabilized cells. Under optimum conditions for oligosaccharide transport we were neither able to detect the release of glycoproteins from the permeabilized cells nor were able to detect Golgi-type processing of oligosaccharides N-linked to glycoprotein despite the fact that we showed Golgi-a-mannosidase I to be functional in the permeabilized cells. Accordingly as the conditions in which free oligosaccharides were transported out of the permeabilized cells into the incubation medium did not permit vesicular transport of glycoproteins from the ER to the Golgi apparatus our data demonstrate the presence of a transport process for the delivery of free polymannose oligosaccharides from the ER to the cytosol.

S8. 11.40am

Cytosolic $Man_5GlcNAc_1$ [Manα1-2Manα1-2Manα1-3(Manα1-6)Manβ1-4GlcNAc] as Degradation Final Product of Oligomannosides Released During N-glycosylation

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Oligosaccharide-phosphates, neutral oligosaccharides possessing one ($OSGn_1$) or two ($OSGn_2$) GlcNAc at the reducing end have been described to be released during the N-glycosylation process in various biological models and, in our case, in CHO and Man-P-Dol deficient CHO mutant cells.

It has been demonstrated that oligosaccharide-phosphates originate from the cleavage by a specific pyrophosphatase of non-glucosylated cytosolic faced oligosaccharides-PP-Dol and chiefly the $Man_5GlcNAc_2$-PP-Dol. The $Man_5GlcNAc_2$-P is recovered in the cytosolic compartment and is further degraded to $Man_5GlcNAc_1$ by not yet depicted enzymes.

In contrast $OSGn_2$ produced from hydrolysis of oligosaccharide-PP-Dol (presumably as a transfer reaction on to water) when the amount of protein acceptor is limiting, are generated into the lumen of rough ER. They are further submitted to processing α-glucosidases and rough ER mannosidase and are (mainly as $Man_8GlcNAc_2$) exported into the cytosolic compartment. There, the $OSGn_2$ is transformed into $OSGn_1$ by a cytosolic chitobiase. This chitobiase action appears as a prerequisite to further activity of cytosolic mannosidase(s) to give the $Man_5GlcNAc_1$: [Manα1-2Manα1-2Manα1-3(Manα1-6)-Manβ1-4GlcNAc] as final product.

In addition pulse chase experiments showed that Man_5-$GlcNAc_1$ is still produced after oligosaccharide-phosphates and $OSGn_2$ have been catabolized (after 1 h chase), indicating an additional source of this product. We demonstrated that Man_5-$GlcNAc_1$ also originated from the degradation of newly synthesized glycoproteins in the rough ER or a closely related compartment. The sequence of events and related ER enzymes involved in this glycoprotein degradation process are under investigation.

S8 POSTERS

S8

Disturbances in the Dolichol-linked Pathway and in Antioxidant Metabolism in Human Arteriosclerotic Vessels

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Lipids play a central role in the genesis of arteriosclerosis. Several publications show that the early steps in the arteriosclerotic process are caused by disturbances in lipid and lipid-protein metabolism within or close to the vessel wall. The accumulation of the so called low density lipoproteins (= LDL) in a modified (i.e. oxidized) form is an important event in the process; since this facilitates the initiation or even 'triggers' the whole arteriosclerotic process. The LDL-particles circulating in the blood are, involved in transport and metabolism of various lipids, and it is suggested by several groups that they are influenced by chemical 'stress' of different kind i.e. oxygen-free radicals. In studies where chemicals have been used, which have antioxidative properties, LDL particles have been protected against oxidation. This has led to a slowing of the arteriosclerotic process. Preliminary data from my study of human vessels have shown altered lipid levels in the vessel wall; indicating a disturbed lipid metabolism in arteriosclerotic vessels. The biological material was obtained from two groups; human normal and arteriosclerotic aortic vessels. After preparation, homogenization, extraction and isolation on HPLC, the various lipids were quantitated. Some lipids were further processed after which fatty acids were studied by GC-technology. In arteriosclerotic vessels the antioxidant ubiquinone (Q10) = coenzyme Q clearly seems to increase (the major part is in a reduced form), which supports the idea of an increased demand during the oxidative stress. Dolichol levels are slightly decreased while dolichol-esters were moderately increased. The dolichol pattern (i.e. the composition of polyisoprenes of various length) was not altered when the normal and arteriosclerotic regions were compared. Cholesterol- and cholesterolesters are dramatically increased, whereas phospholipids showed a moderate increase and a relative increase of sfingomyelin and phosphatidylcholine when compared to normal. The fatty acid pattern in individual phospholipids showed modest alterations when the two groups were investigated. The study shows that lipids in the mevalonate pathway and dolichol-linked pathway are involved indirectly or directly in the arteriosclerotic process.

S8

Mannosyl-P-Dolichol Stimulates the Formation of GlcNAc-P-P-Dolichol but not $(GlcNAc)_2$-P-P-Dolichol

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Previous studies from this laboratory have revealed that man-P-dol acts as an allosteric activator of the initial reaction of the dolichol pathway [1]. However, since the conversion of GlcNAc-P-P-dol to $(GlcNAc)_2$-P-P-dol is a sequential reaction, stimulation of the former will also lead to an increased formation of the latter. Further studies were carried out to investigate the direct site of the stimulatory effect of man-P-dol. The rationale was to examine the influence of the activating compound on the synthesis of $(GlcNAc)_2$-P-P-dol using GlcNAc-P-P-dol synthesized *in situ* as the substrate. A two phase incubation protocol was followed in which microsomes from the retina of the embryonic chick were incubated during the first phase in the presence of a complete GlcNAc-lipid synthesizing system using non-radioactive UDP-GlcNAc as a substrate. During the second phase UDP[^{3}H]GlcNAc was added and the incubation continued in the presence or absence of tunicamycin and the presence or absence of man-P-dol. In the presence of tunicamycin the only radioactive $(GlcNAc)_2$-P-P-dol that would be formed during the second phase would be those molecules generated by the addition of [^{3}H]GlcNAc to non-radioactive GlcNAc-P-P-dol formed during the first phase. By comparison with appropriate controls, the effect of man-P-dol on the synthesis of $(GlcNAc)_2$-P-P-dol was discernible. Consistent with previous studies, these studies demonstrated that the stimulation of GlcNAc-lipid synthesis by man-P-dol was due to the enhanced synthesis of GlcNAc-P-P-dol and not to the activation of the GlcNAc-transferase concerned with the attachment of the second GlcNAc residue for the biosynthesis of the chitobiosyl derivative.

Supported in part by NIH grant EY00393.

1. Kean EL (1985) *J Biol Chem* **260**: 12561–71.

S8

Negative-Acting Element Involved in the Hormonal Regulation of GlcNAc-1-P Transferase Gene

J. Ma[1], H. Saito[2], T. Oka[2] and I. K. Vijay[1]
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[2] *Laboratory of Molecular and Cell Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Health, Bethesda, MD 20892, USA.*

The gene encoding UDP-GlcNAc:dolichol phosphate *N*-acetylglucosamine-1-phosphate transferase (GPT), the enzyme that initiates the pathway for the biosynthesis of asparagine-linked glycoproteins, is developmentally and hormonally regulated in mouse mammary gland. Transcription of the *GPT* gene is regulated by the lactogenic hormones insulin, glucocorticoid, and prolactin. The *cis*-acting elements involved in regulating the expression of the gene have been investigated by transient transfections of primary mouse mammary epithelial cells. The 1055 bp 5′-flanking sequence of GPT promoter is sufficient for hormonal induction of a luciferase reporter gene construct. A series of 5′-deletions of the GPT promoter region identified a distal repressor element (−1055 to −966 bp). Deletion of the distal repressor element results in enhanced hormonal induction (~seven-fold) with no effect on basal promoter activity. Electrophoretic mobility shift assays performed with nuclear extracts from different developmental stages of mouse mammary gland demonstrated that the binding activity to the distal element was predominantly observed in virgin stage as compared with pregnant and lactating stages. These findings

provide a basis to elucidate the molecular mechanism of *GPT* gene expression by hormones in the mammary gland.

Supported by N.I.H. grant DK 19682.

S8

Microanalyses of Dolichyl Derivatives and the GlcNAc-1-phosphate Transferase Activity of Carbohydrate-Deficient-Glycoprotein (CDG) Syndrome

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Sequential microanalyses of free dolichol, dolichyl fatty acid ester and dolichyl phosphate were made. This method is simple and three types of dolichols can be estimated using the same HPLC system after the derivatization with anthracene-9-carboxylic acid. Using this method, dolichols and their derivatives were determined in the fibroblasts from patients with CDG syndrome. The contents of total dolichols, dolichyl phosphate and the activity of the UDP-*N*-acetylglucosamine-:dolichyl phosphate *N*-acetylglucosamine 1-phosphate transferase (GlcNAc-1-P transferase) of patients' fibroblasts were similar to those in normal fibroblasts. These results suggest that CDG syndrome may not be due to a deficiency of a biosynthetic enzyme for dolichol-oligosaccharide intermediates but to a metabolic error in assembly of asparagine-linked oligosaccharide.

S9. SYNTHESIS AND DEGRADATION OF GLYCOSPHINGOLIPIDS

Chairs: Robert Yu, Subhash Basu

S9. 9.50am

The Defect in Biosynthesis of Glycosphingolipids (GSLs) in SW13 Cells Lacking Intermediate Filaments (IF) is in Recycling and not in De novo Biosynthesis

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Our previous observations [1, 2] on the immunocytochemical colocalization of GSLs and IF of the cytoskeleton led us to analyse the role of IF in the biosynthesis and intracellular transport of GSLs. Cells with and without an IF network (IF^+ and IF^-) were cloned from the human adrenal carcinoma cell line SW13. Metabolic labelling experiments showed that IF^- cells incorporate less sugar into GSLs, especially LacCer and Gb3Cer, than IF^+ cells, and this abnormality can be reversed by expression of a mouse vimentin network in the IF^- clones [3]. Recent data indicate that the defect in sugar incorporation occurs in the endocytic recycling pathway. Metabolic double-labelling studies showed differences between IF^+ and IF^- clones in sugar incorporation, but not in serine incorporation. These data indicate that there is no difference in *de novo* biosynthesis, and that the abnormality is in sugar incorporation into recycling GSLs. We confirmed this conclusion by use of β-chloroalanine, which inhibits *de novo* synthesis of sphinganine, and fumonisin, which inhibits both acylation of sphinganine and reacylation of sphingosine generated by catabolism of sphingolipids. We are now investigating the mechanism by which lack of an IF network interferes with the recycling of GSL.

Supported by grants from the American Cancer Society (BE-63173) and the National Institutes of Health (AI 17712).

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2. Gillard BK *et al.* (1992) *Cell Motil Cytoskeleton* **21**: 255–71.
3. Gillard BK *et al.* (1994) *J Cell Science* **107**: 3545–55.

S9. 10.05am

Ganglioside Biosynthesis in the Mouse Embryo: A Developmental Study

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Gangliosides are acidic glycosphingolipids that are enriched in the plasma membrane of neurons. There is considerable evidence to suggest that gangliosides play a significant role during the development of the vertebrate embryo. We have previously reported use of whole embryo culture (WEC) to analyse ganglioside biosynthesis in postimplantation mouse embryos. In this study, we compared ganglioside biosynthesis of early embyros (5–6 somites) and late embryos (26–28 somites). The early embryos were cultured for 2 h prior to the addition of ^{14}C-galactose to the culture medium while the late embryos were cultured for 40 h prior to radiolabel addition. Both early and late embryos were exposed to ^{14}C-galactose for 6 h and then sacrificed. The concentration of ^{14}C labeled gangliosides (dpm/μg protein) was 31.3 and 15.2 ± 2.2 in the early and late embryos, respectively. Furthermore, the percentage distribution of the major gangliosides synthesized by the early embryo (GM3, GM1, GD3 and GD1a) was remarkably similar to the distribution of these gangliosides in the late embryo. This is intriguing since the early and late embryos undergo totally different morphological stages during the respective 6 h labelling periods: the early embryos have open neural tubes and rudimentary cardiac tissues and the late embryos have closed neural tubes with 5 brain vesicles as well as a heart that beats 120 times per min. Our results suggest that a constant, stable or invariant ganglioside distribution may be important during early stages of mammalian morphogenesis.

S9. 10.20am

Isolation of Putative cDNA Clones for SAT-3 from Embryonic Chicken Brain (ECB) and Human Colon Carcinoma (Colo 205) Cell

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A novel sialyltransferase, SAT-3 activity (CMP-NeuAc:nLc-Ose4Cer α2-3 sialyltransferase), was characterized in ECB [1]. Recently, a sialyltransferase cDNA from human placenta [2] has been cloned and expressed with similar enzymatic properties. To test the genetic similarity between these two enzymes,

we employed a reverse transcription polymerase chain reaction (RT-PCR) based strategy to isolate cDNA clones from ECB and Colo 205 cell. Two pairs (Set I and Set II) of PCR primers were designed from the human placenta SAT-3 cDNA sequence. The sequences of Set II primers nest within the ~800 bp cDNA region spanned by Set I primers. The 5′end primer of Set II was selected from the region coding for Sialylmotif, and the 3′end primer (Set II) was selected from the second highly conserved region among the sialyltransferases. RT-PCR with primer Set I amplified ~800 bp cDNA fragments from ECB and Colo 205 RNA. These ~800 bp cDNAs hybridized with human placenta SAT-3 probe on Southern blotting analysis. When the ~800 bp cDNA fragment was used as template for PCR analysis with Set II primer pair a ~400 bp DNA fragment was produced, as expected. Both ~800 and ~400 bp cDNA fragments, from ECB and Colo 205, were cloned into pT7-Blue vector (Novagen) providing single 3′T overhangs at insertion site. Sequence analysis of the ECB cDNA showed a high level of nucleotide sequence homology (>90%) with the SAT-3 cDNA from human, and the presence of the Sialylmotif coding region. Sequencing of the Colo 205 cDNA is in progress.

Supported by NIH-18005 Jacob Javits Award to S.B.

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S9. 10.35am

Accumulation of Endogenous GM3 by Alteration of Glycolipid Synthesis with Antisense Oligonucleotides to Glycosyltransferases in HL-60 Cells: Effects on Cellular Differentiation

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Synthetic oligodeoxynucleotides antisense to human GM2-synthase and GD3-synthase were used to treat the human promyelocytic leukaemia HL-60 cells, resulting in a dramatic reduction (about 80%) of the activity of GM2 and GD3 synthases. Oligodeoxynucleotides sense to GM2- or GD3-synthase failed to alter the expression of GM2 or GD3 synthase. Analysis of the ganglioside composition of HL-60 cells showed that suppression of GM2- and GD3-synthases by the antisense oligomers led to the accumulation of the common precursor GM3 of the two enzymes. Accumulation of GM3 was also achieved by TPA induction in which the level of GM3 increased remarkably, presumably by stimulation of the activities of GM3-synthase. When HL-60 cells were treated with GM2-synthase antisense oligomer alone, both GM3 and GD3 increased; the increase in GD3 was most likely due to the conversion of the accumulated GM3 to GD3 when the 'a-pathway' of ganglioside biosynthesis was blocked. The treated cells underwent monocytic differentiation as judged by morphological changes, adherent ability, and nitroblue tetrazolium (NBT) staining. Since the common feature of the induced cellular differentiation in HL-60 cells either by TPA, exogenously added GM3, and the antisense oligonucleotides observed in this present study is a remarkable increase in cellular GM3 concentrations, it suggests that the increase in endogenous GM3 may be associated with the cellular differentiation process. The antisense DNA technique used in this study may prove to be a powerful tool in manipulating glycolipid synthesis in the cell.

S9. 10.50am

O-Acetylesterase Gene Down-regulates the Expression of *O*-Acetylated GD3 in a Neuroblastoma Cell Line, F-11

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Previous studies from our laboratory revealed that the mouse F-11 neuroblastoma cell line, which is originated from hybridization of N18TG-2 (mouse neuroblastoma) with rat dorsal root ganglion cells, had the following ganglioside composition: GM3 (37% of the total gangliosides), GD3 (27%), *O*-acetylated GD3 (18%) and GD1a (4%) with trace amounts of GD2 ganglioside. Immunocytochemical localization of GD3 and *O*-acetylated GD3 examined by mouse monoclonal antibodies R24 and D 1.1, respectively, revealed that the cell bodies and neurites were positively stained by both antibodies. To study the functional role of *O*-acetylated GD3, we transfected F-11 cells with *O*-acetylesterase gene from influenza C virus which resulted in an increase of GD3 (150% of control cells) and a decrease of *O*-acetylated GD3 (27% of control cells). The transfected F-11 cells appeared to be more differentiated, e.g. enlarged cell bodies and elongated neurites. We concluded that alteration of ganglioside expression, particularly *O*-acetylated GD3, may be associated with neuronal differentiation in this cell line. The study also provides direct evidence that cellular ganglioside expression can be manipulated by transfection of the cell with a foreign gene and this approach may represent a powerful means in elucidating the function of gangliosides.

S9. 11.05am

Novel Inhibitors of Glycosphingolipid Biosynthesis

Frances M. Platt[1], Gabrielle Neises[2], Raymond A. Dwek[1] and Terry D. Butters[1]
[1]*Glycobiology Institute, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK.*
[2]*Monsanto Co., St Louis, MO 63198, USA.*

We have recently discovered that the imino sugar *N*-butyldeoxynojirimycin (*N*B-DNJ) inhibits glycolipid biosynthesis *in vitro*, in addition to its known activity as an inhibitor of the N-liked oligosaccharide processing enzymes α-glucosidase I and II. We have also established that glycolipid biosynthesis inhibition occurs *in vivo* by administering *N*B-DNJ to mice. We observe a dose-dependent decrease in cell surface glycosphingolipid levels on tissues derived from treated animals.

In an attempt to dissociate the two enzyme inhibitory activities of *N*B-DNJ and to identify an inhibitor which was more selective for the glycolipid biosynthetic pathway, several imino sugars have been *N*-alkylated and tested for inhibitory activity. The galactose analogue *N*-butyldeoxygalactonojirimycin (*N*B-DGJ) was found to be a potent inhibitor of glycolipid biosynthesis but in contrast to *N*B-DNJ had no effect on the maturation of N-linked oligosaccharides or on lysosomal glucocerebrosidase. The effect of increasing N-alkyl chain length on glycolipid inhibition was investigated. Non-alkylated DGJ, the

N-methyl and N-ethyl derivatives were non-inhibitory. However, N-propylation resulted in partial inhibition while the N-butyl and N-hexyl derivatives resulted in maximal inhibition. Increasing alkyl chain length also resulted in increased potency of glucosyltransferase inhibition.

In an *in vitro* Gaucher's disease model *N*B-DGJ was as effective as *N*B-DNJ in preventing glycolipid storage and may represent a more selective potential therapeutic agent than *N*B-DNJ for the management of this and other glycosphingolipidoses.

S9. 11.20am

Modulation of Neuroblastoma Ganglioside Synthesis and Shedding by PDMP

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Shedding of tumour cell surface gangliosides, which have potent immunosuppressive activity, is a potential mechanism contributing to tumour cell escape from the host immune response. Engineering of the cell to block the synthesis and prevent the shedding of gangliosides would thus be important. PDMP is a potent inhibitor of glucosylceramide synthase, and it inhibits the synthesis of cellular glycosphingolipids. When human neuroblastoma LAN-5 cells were treated with 10 μM PDMP for 24 h, cellular ganglioside content decreased to 50% while the metabolic radiolabelling rate of gangliosides was more rapidly reduced, to 20% of that of the control cells. Studies of the gangliosides shed by PDMP-treated cells show that the rate of shedding is linked to the rate of ganglioside synthesis, which is reduced by PDMP. Finally, when LAN-5 cells were treated with 20 μM PDMP for 5 days, both the rate of ganglioside synthesis and the cellular ganglioside content were reduced to less than 10% of control values. Shedding of gangliosides by tumour cells under these conditions was almost completely abolished. Since both synthesis and shedding of gangliosides can be significantly downregulated by PDMP, it will be important to study the biological effect of PDMP on neuroblastoma ganglioside shedding *in vivo*.

Supported by NCI grant CA61010.

S9. 11.35am

Ceramide Glycanase from Rat Mammary Tissue

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Ceramide glycanase (CGase), the enzyme which cleaves the oligosaccharide chain of a glycosphingolipid and hydrophobic ceramide moiety, has been characterized previously from bacteria, leech, clam and earthworm. Recently, purified clam CGase has been used to analyse various glycolipid structures with the use of FACE [1]. Previously, we have reported characterization of CGase from rabbit mammary tissue [2]. Mammalian ceramide glycanase might play a significant role in signal transduction. The cleavage product ceramide, could be further cleaved to sphingosine or its analogue, a modulator in the signal transduction pathway. The modulatory effect of sphingosine on glycolipid: glycosyltransferases is also observed *in vitro* [3]. We report here the characterization of ceramide glycanase in pregnant as well as in lactating rat mammary tissue. The activity is distributed in soluble supernatant (100 000 $\times$g) and Golgi-rich membrane. Presence of detergent seems to be crucial for optimal enzyme activity. Preliminary studies indicate that the enzyme activity is exhibited over a pH range between 5.0 and 6.0 in citrate-phosphate buffer at a lower detergent concentration (0.5%), both soluble enzyme and Golgi-rich membrane fraction. However, at a higher detergent concentration (1.0%), a sharp pH optimum of 5.0 is observed for Golgi-rich membrane. Among various glycolipids tested, GM1 and Gg4 seem to be the best substrate for the rat mammary CGase. The presence of the enzyme has also been detected in the rat mammary tissue at various stages of gestation and during lactation period.

Supported by NS-18005 to S.B.

1. Basu S *et al.* (1994) *Anal Biochem* **222**: 270–74.
2. Basu S *et al.* (1990) *Ind J Biochem Biophys* **27**: 386–95.
3. Das *et al.* (1990) *IJBB* **27**: 396–401.

S9. 11.50am

Regulation of Soluble Sialidase Activity in Chinese Hamster Ovary (CHO) Cells Using Antisense RNA

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We have previously characterized a soluble sialidase in the culture fluid of Chinese hamster ovary cells that alters the sialic acid composition of recombinant glycoproteins expressed in this host [1]. In order to reduce sialidase levels in CHO cells we have established stable transfectants containing antisense RNA derived from the sialidase gene [2]. Constructs were made by inserting the inverted orientation of the intact sialidase cDNA (1.4 kb), and cDNA segments from the 5′ untranslated region (189 bp), the 5′ coding region (474 bp), and the 3′ coding region (686 bp), into SV40-based expression plasmids. Cotransfections with a vector providing puromycin resistance as a selectable marker were carried out using both linearized and non-linearized antisense constructs. Screening of 50 clones gave two isolates, AS474#16, and AS686#3 with sialidase activity reduced 50% and 60%, respectively, over wildtype levels. Immuno blot analysis of cell extracts confirmed the substantial reduction of sialidase protein.

1. Warner TG *et al.* (1993) *Glycobiology* **3**: 455.
2. Ferrari J *et al.* (1994) *Glycobiology* **4**: 367.

S9 POSTERS

S9

Purification and Characterization of PAPS:GalCer Sulfotransferase from Human Renal Cancer Cells

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We purified PAPS:GalCer sulfotransferase (EC 2.8.2.11) from the human renal cancer cell line SMKT-R3 through a combination of affinity chromatographies using galactosylsphingosine, 3′,5′-bisphosphoadenosine and heparin as ligands. The purified sulfotransferase showed a specific activity of 1.2 μmol-min^{-1} mg^{-1}, which was 300 times more than the highest activity among the enzyme preparations purified so far from other sources. Homogeneity of the purified sulfotransferase was

assessed by the fact that the enzyme preparation showed a single protein band with an apparent molecular mass of 54 kDa on reducing SDS-PAGE and that protein bands coincided with the enzyme activity both on native PAGE and nonreducing SDS-PAGE. GalCer was the best acceptor for the purified enzyme. LacCer, GalAAG and GalDG were also good acceptors. GlcCer, Gg3Cer, Gg4Cer, Gb4Cer, Lc3Cer and nLc4Cer did serve as acceptors although the relative activities were low. On the other hand, the enzyme could not act on Gb3Cer, which possesses α-galactoside at the nonreducing terminus. Neither galactose nor lactose served as an acceptor. These observations suggest that the sulfotransferase prefers β-glycoside, especially β-galactoside, at the nonreducing termini of sugar chains attached to a lipid moiety.

S9

Enhancement of Cerebroside Sulfotransferase Activity in MDCK Cells by Excess NaCl

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Madin-Darby canine kidney (MDCK) cells respond to kidney-oriented hormones and synthesize sulfoglycolipids. A single enzyme catalyses the transfer of sulfate to both GalCer and LacCer [1, 2]. Turnover of sulfoglycolipids was enhanced in the medium made hypertonic by adding NaCl or mannitol to 440 and 580 mOsmol l^{-1} [3] suggesting that the activity of the sulfotransferase is increased.

MDCK cells were cultured to confluency in an EMEM-based medium containing 10% fetal bovine serum and further maintained in a medium containing 1% FBS for 2 days to minimize the effect of growth factors in the serum. Then the medium from a set of dishes was supplemented with 100 mM NaCl. After 24 h in the hyperosmolar medium, the incorporation of [^{35}S]sulfate into sulfated glycolipids (galactosyl- and lactosyl sulfatides) was 1.3–1.5-fold higher than the control.

The sulfotransferase activity was assayed using GalCer as the acceptor substrate by the method described previously [2] with slight modifications. The activity was elevated 3.6-fold after 24 h of the stimulation with excess NaCl ($p < 0.01$). This increment was comparable to that by 10% serum. In conclusion, the ability of sulfoglycolipids in MDCK cells to adapt to hyperosmotic environments may involve the increase of sulfotransferase activity.

1. Ishizuka I, *et al.* (1978) *Biochim Biophys Acta* **541**: 467.
2. Tadano K, Ishizuka I (1979) *Biochim Biophys Acta* **575**: 421.
3. Niimura Y, Ishizuka I (1990) *Biochim Biophys Acta* **1052**: 248.

S9

Molecular Cloning and Expression of Endoglycoceramidase Gene of a *Rhodococcus* sp.

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Endoglycoceramidase (EGCase; EC 3.2.1.123) cleaves the linkage between oligosaccharides and ceramides of various glycosphingolipids [1]. Three molecular species of EGCase, I-III, each with its own specificity, have been isolated from the culture supernatant of a *Rhodococcus* sp. [2]. In this study, we isolated a gene encoding EGCase II of *Rhodococcus* sp. strain M-777 and expressed it in *Escherichia coli*. We synthesized probe DNAs based on internal amino acid sequences of purified EGCase II. Chromosomal DNA was digested with three restriction enzymes followed by Southern hybridization with the synthesized DNA probes. The gene encoding the EGCase II was in this way screened for by colony hybridization from colonies of cells transformed with size-fractionated DNA. An open reading frame of the *EGCase II* gene encoded a polypeptide of 490 amino acids. The amino acid sequence deduced from the nucleotide sequence was similar to a conserved region in the active site of bacterial endo-β-glucanases. Therefore, EGCase II was an endoglycosidase, not a lipase. When the *EGCase II* gene was subcloned into the expression vector pTV118N, which was used to transform *E. coli* JM109 cells, EGCase II activity appeared in the cell extract. No contaminating sphingomyelinase activity was found in the cell extract.

1. Ito M, Yamagata T (1986) *J Biol Chem* **261**: 14278–82.
2. Ito M, Yamagata T (1989) *J Biol Chem* **264**: 9510–19.

S9

Molecular Cloning and Expression of the Gene Encoding an Activator of Endoglycoceramidase Activity

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Endoglycoceramidase (EGCase; EC 3.2.1.123) cleaves the glycosidic linkage between the oligosaccharide and ceramide of various glycosphingolipids [1]. Protein activators that stimulate EGCase activity in the absence of detergents have been purified from the culture supernatant of a *Rhodococcus* sp. [2]. There are two molecular species of activators; activator I stimulates the activity of EGCase I and activator II stimulates that of EGCase II. In this study, we cloned the activator II gene of *Rhodococcus* sp. strain M-777 and expressed it in *Escherichia coli*. Degenerate DNAs were synthesized on the basis of amino acid sequence data for purified activator II and used as primers to amplify fragments of the gene from chromosomal DNA of *Rhodococcus* sp. strain M-777 by the polymerase chain reaction. One of the amplified fragments was used as a probe to screen for the gene encoding activator II by colony hybridization. When the gene was inserted into an expression vector, pET23, and *E. coli* JM109 cells were transformed with the vector, activator II protein was found in the cell extract. As for native activator II protein [3], activator II protein expressed in *E. coli* was converted by trypsin into a still fully active polypeptide with the molecular weight of 27 900.

1. Ito M, Yamagata T (1989) *J Biol Chem* **264**: 9510–19.
2. Ito M, Ikegami Y, Yamagata T (1991) *J Biol Chem* **266**: 7919–26.
3. Ito M, Ikegami Y, Omori A, Yamagata Y (1991) *J Biochem* **110**: 328–32.

S9

Synthesis *in vitro* of Blood Group H1 Antigen and Fucosyllactosylceramide by α 1-2 Fucosyltransferase from PC12 Cells

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Neurite outgrowth of PC12 cells induced by nerve growth factor (NGF) is associated with changes in glycosyltransferase levels, accompanying the alteration in the composition of glycosphingolipids including a blood group B antigen. Previously, we examined the expression of GDP-fucose:glycosphingolipids fucosyltransferase activity of PC12 cells in relation to the differentiation by NGF. This enzyme was found to be most active for nLc4 (paragloboside) followed by LacCer (Lactosylceramide). In this study we have identified the products formed by the fucosyltransferase(s) for nLc4 and LacCer in PC12 cells. Transfer of ^{14}C-fucose to nLc4 yielded a labelled product having lower mobility than nLc4 on TLC. The product was determined to be a blood group H1 antigen by GC/MS analysis, suggesting that PC12 cells have α1,2 fucosyltransferase (α1,2-FT) which catalyses the biosynthesis *in vitro* of blood group H1 antigen from nLc4. In addition, the findings evidence the presence of a biosynthetic precursor of B antigen in PC12 cells. ^{14}C-Fucose was also incorporated into LacCer, forming glycolipids comigrating with globotriaosylceramide on TLC. This is the first report on the presence of α1,2-FT in cultured neuronal cells.

S9

Manipulation of Glycosphingolipid Biosynthesis by Artificial Glycolipids [I]

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This study is conducted to develop artificial well-designed glycolipids by which the role of endogenous glycosphingolipids (GSLs) will be elucidated. Should they be successfully settled in the Golgi apparatus and recognized by glycosyltransferase(s), elongation of the given artificial glycolipid will occur followed by the possible disturbance of the functional distribution of endogenous GSLs in cells, direct functions of endogenous GSLs will be revealed.

We have prepared amphiphilic lactosides possessing varying lengths of the alkyl or acyl chain. Using a panel of the lactosides, we found that the artificial lactosides functioned as a saccharide acceptor for glycosyltransferase in the cell. Artificial lactosides with a shorter hydrophobic chain, e.g. C8, were readily incorporated into mouse neuroblastoma and melanoma cells where the addition of the saccharide unit occurred followed by secretion of them into the culture medium. On the other hand, lactosides bearing longer chain, e.g. C16, were settled in the plasma membrane. Cultivation of the cell with each of lactosides affected cell morphology and growth in a chain length or concentration dependent manner, suggesting the importance of the structures of hydrophobic chain in their targeting. Lactosides were also recognized by glycosyltransferases in cell homogenates producing ganglioside-like structures which were not found in intact cells. This suggests a possible use in detection and searching related enzyme activity. The capacity of the artificial glycolipids to modify biological response in cells is now being investigated in detail.

S9

Isolation and Analysis of Human Melanoma Cells Deficient in GD3 Expression

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Treatment of human melanoma cell line SK-MEL-28 with anti-GD3 antibody (R24) and rabbit complement and subsequent sub-cloning of the surviving cells resulted in the derivation of two cell lines deficient in the cell surface expression of GD3. Both cell lines (designated SK-MEL-28-N1 and SK-MEL-28-N2) had no detectable cell surface expression of GD3 as analysed by a mixed haemagglutination assay with mAb R24. No GD3 was detectable in either cell line by glycolipid isolation, TLC and resorcinol-HCl spray. However, TLC-immunostaining with mAb R24 showed the presence of low amounts of GD3 in both N1 and N2 (1/40th of the amount in the parent cell line in N1 and 1/500 in N2). In SK-MEL-28-N1 the residual GD3 was shown by immunofluorescence assays on permeabilized cells to be present in discrete intracellular organelles, suggesting a defect in transport as well as in synthesis. Both SK-MEL-28-N1 and -N2 had an increase in detectable GM3 expression. The mutant cell lines had altered cell morphology in comparison to the parent cell line and both had slower growth rates *in vitro* and N1 did not grow in nu/nu mice (N2 has not yet been tested). These cell lines will be useful in exploring the biological function of GD3 ganglioside.

S9

Molecular Cloning of a cDNA(34A34) That Increases the Expression of Galactosylceramide in COS-7 Cells

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[2]*Advanced Institute of Science and Technology, Nara, Japan.*

We have attempted to isolate a UDP-galactoside ceramide galactosyltransferase cDNA clone by a transient expression method, and isolated one cDNA clone(34A34). Transfected COS cells with the 34A34 cDNA expressed the galactosylceramide, which was determined by FACS analysis and radioactively metabolic labelling. The galactosyltransferase activity, was not however, clearly detected in the transfected COS cells. The cDNA encodes a polypeptide of 696 amino acid residues with a molecular weight of 77276. The polypeptide has two proline-rich domains and the proline residues amount to 18% of amino acids in the C terminus proline-rich domain. The cDNA 34A34 hybridized to a single mRNA of 3.1 kb in all rat organs examined. The mRNA was expressed mostly in the testis. Shulte and Stoffel [1] recently isolated a protein and a cDNA of the UDP-galactoside ceramide galactosyltransferase. Their cDNA and amino acid sequence do not exhibit any similarities with our cDNA 34A34 or the one sequence, respectively. The 34A34 cDNA, therefore, may not be the cDNA of the UDP-galactoside ceramide galactosyltransferase. We are seek-

ing possible roles of the protein encoding the cDNA 34A34 in the expression of galactosylceramide.

1. Schulte S, Stoffel W (1993) *Proc Natl Acad Sci* **90**: 10265–69.

S9

Partial Purification of Glucosylceramide Synthase from Rat Liver Golgi Membranes

P. Paul, Y. Kamisaka, D. Marks and R. E. Pagano
Mayo Clinic, 200 First Street, SW, Rochester, MN 55905, USA.

Glucosylceramide synthase catalyses the biosynthesis of glucosylceramide, a major precursor of higher-order glycosphingolipids. This enzyme was solubilized and partially purified from an enriched Golgi-membrane fraction isolated from rat liver. The membrane fraction was first washed with an anionic (*N*-lauroylsarcosine) detergent and the enzyme was subsequently solubilized in the presence of CHAPSO. Purification was achieved through a two step dye affinity chromatography procedure. Golgi components such as sphingomyelin synthase bound to the dye-agarose while most of the glucosyltransferase activity was recovered in the flow through in the presence of the substrate UDP-Glc. After a gel filtration step to remove UDP-Glc, the glucosylceramide synthase was purified with an apparent enrichment of about 5000-fold relative to the homogenate by rechromatography on a dye-agarose column using UDP-Glc for elution. The substrate specificity of the glucosylceramide synthase was assessed by using either fluorescent or radioactive ceramide analogues. We found that the activity of the partially purified enzyme was stereospecific and dependent on the N-alkyl chain length as well as on the substituting fluorophore of the ceramide. In addition, only UDP-Glc was used as a substrate by the enzyme among a variety of other UDP-
sugars. The partially purified enzyme was further characterized by sedimentation through a glycerol gradient.

Our long term goals are to understand how the activity of this molecule is regulated within the cell and to learn what targeting signals are responsible for its localization at the Golgi complex.

Supported by USPHS Grant R37 GM-22942.

S9

Expression of α1,3 Fucosyltransferase in Developing Rat Brain

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E. K. Shriver Center, 200 Trapelo Road, Waltham, MA 02254 and Harvard Medical School, Boston, MA, USA.

SSEA-1(Le^x) antigens, containing 3-fucosyl-*N*-acetyllactosamine epitope, are present on cells and play an important role in intercellular communication. Fucosyltransferases (Fuc-T) that catalyse the final step(s) in the biosynthesis of SSEA-1 probably play a critical role in the expression of these antigens and have not yet been characterized in the CNS. We studied Fuc-T activity in rat forebrain and cerebellum at different stages of development. Oligosaccharides (lactose, *N*-acetyllactosamine) and glycolipid (paragloboside, nLc_4), were used as acceptors. Triton X-100 soluble fraction of forebrain and cerebellum was used to assay for enzyme activity at pH 7.4. Our results indicate that Fuc-T activity in forebrain from 17 day old rats was five times higher with lactosamine (acceptor for Fuc-T IV) than that obtained with lactose (acceptor for Fuc-T III, V and VI). Isoelectric focusing profile of the Fuc-T activity obtained in presence of lactose was different from that in presence of *N*-acetyllactosamine and nLc_4. In cerebellum from rats at E20 through P100 days the maximal enzyme activity was obtained at P15 with both acceptors. The radioactive fucosylated enzymatic product with nLc_4 as the acceptor was SSEA-1 positive with 7A antibody indicating that the enzyme product was fucosylated α1,3 nLc_4. Therefore, the major α1,3 Fuc-T in rat cerebellum is an α1,3 fucosyltransferase with properties similar to those reported for human fucosyltransferase IV.

This study was supported by NIH grants HD05515 and NS15037.

MONDAY 21 AUGUST, AFTERNOON

PLENARY LECTURES

S10. 2.40pm

Plants Utilize Many Oligosaccharin Signal Molecules

Peter Albersheim, Kyung-Sik Ham, Carl Bergmann, Stefan Eberhard and Alan Darvill
Complex Carbohydrate Research Center and Department of Biochemistry and Molecular Biology, University of Georgia, 220 Riverbend Road, Athens, GA 30602-4712, USA.

Carbohydrates, the building blocks of structural polymers that give form to living cells and organisms, also play important roles in the interactions of cells with one another and with other organisms. Plants have evolved signalling pathways to regulate the expression of genes essential to their growth, development, and interactions with symbionts and pathogens. Some of the signal molecules in each of these pathways are oligosaccharides that are collectively known as oligosaccharins. This overview lecture discusses: (i) the exquisite structural specificity required for oligosaccharin activity; (ii) the high degree of host specificity exhibited by oligosaccharins required for symbiosis; (iii) the different functions for the same oligosaccharin in different plant species; and (iv) the critical role of oligosaccharins in the highly evolved, multilevel interactions exhibited by plants and their pathogens.

Research supported by US Dept of Energy (DOE) grant DE-FG05-93ER20114 and the DOE-funded (DE-FG09-93ER20097) Center for Plant and Microbial Carbohydrates.

S11. 3.10pm

Mechanism of Golgi Protein Targeting

Graham Warren and Tommy Nilsson
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Golgi proteins are retained in particular compartments by a

retention signal in the membrane-spanning domain and flanking sequences [1]. Some of these proteins also have retrieval signals which return the protein to the compartment after it has left. Several mechanisms have been put forward to explain how these signals operate and these will be discussed.

1. Nilsson T, Warren G (1994). *Curr Opin Cell Biol* **6**, 517–21.

S10. PLANT GLYCOBIOLOGY

Chairs: Alan Elbein, Marilynn Etzler

S10. 4.00pm

Introduction

S10. 4.05pm

A Novel Lectin In The Roots of *Dolichos Biflorus* May Function as a Signal Transducer in Plant-Rhizobial Interaction

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Although legume lectins have long been hypothesized to play a role in determining the specificity of Rhizobial-legume interactions, most work in this area to date has been conducted with lectins found in the seeds of the plants. We have now cloned and sequenced the genomic and cDNA encoding the DB46 lectin present in the roots of the legume, *Dolichos biflorus*. This lectin, which is present at the time and region of the root at which symbiosis occurs, has a unique sequence encoded by nine exons. Although the sequence encoded by the first two exons shows only a faint resemblance to part of the sequence of the seed lectin from this plant, the root lectin resembles the seed lectin in its carbohydrate specificity for α-linked *N*-acetylgalactosamine and reacts with Rhizobia specific for this plant. Antibodies specific for actin react with this lectin, although no sequence similarity with actin has been detected. The results raise the possibility that this lectin may serve as a transducer in linking Rhizobial binding to the cytoskeletal rearrangements that occur as one of the first steps of Rhizobial-legume symbiosis.

Supported by NIH Grant GM21882.

S10. 4.25pm

Identification of a Putative Receptor Protein for *N*-Acetylchitooligosaccharide Elicitor on the Plasma Membrane of Suspension-Cultured Rice Cells by Photoaffinity Labelling

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Department of Cell Biology, National Institute of Agrobiology Resources, Tsukuba, Ibaraki, 305 Japan.

Chitin oligomers of various degree of polymerization (DP) are the fragments of the backbone polymer of the cell wall of many pathogenic fungi and are known to elicit defence responses in several higher plant systems. We already demonstrated that chitin oligomers of DP7–8 ($(GlcNAc)_{7-8}$) could act as a potent elicitor of phytoalexin synthesis in suspension-cultured rice cells even in the nM range [1] and that a high-affinity binding site (order of nM) for the elicitor is present in the plasma membrane [2, 3]. As the first step towards the isolation of the putative receptor protein, we attempted to identify that by photoaffinity labelling. ^{125}I-labelled photolabile aryl azide conjugate of $(GlcNAc)_8$ was synthesized. The plasma membrane prepared by aqueous two-phase partitioning was reacted with the radiolabelled ligand and irradiated with UV light. The membrane was solubilized with SDS, the proteins were precipitated with methanol and were analysed by SDS-PAGE. Autoradiography of the gels showed the labelling of a single 70 kDa band. The 70 kDa band was digested by trypsin which suggests the band is a protein. The incorporation of the ligand into the band showed a saturable mode of binding and was inhibited by unlabelled $(GlcNAc)_8$ (1/2max = 30 nM) but was not inhibited by trimer $(GlcNAc)_3$ nor the deacetylated octamer $(GlcN)_8$. The results are in good agreement with the specificity and the sensitivity for the eliciting activities of the elicitor. This is the first report on the identification of a plasma membrane protein which may function as a receptor for the *N*-acetylchitooligosaccharide elicitor.

1. Yamada A *et al.* (1993) *Biosci Biotech Biochem* **57**: 405.
2. Shibuya N *et al.* (1993) *FEBS Lett* **329**: 75.
3. Ebisu N *et al.* (1994) *Plant Cell Physiol* **35**: S64.

S10. 4.40pm

Plant Glycoprotein Biosynthesis and Intracellular Transport

I. Faye[1], A.-C. Fitchette-Lainé[1] and P. Lerouge[2]
[1]*LTI-CNRS URA 203-IFRMP, Université de Rouen, B.P.118, 76821 Mont Saint Aignan cedex, France.*
[2]*Centre Régional de Spectroscopie, CNRS URA 464-IFRMP, Université de Rouen, B.P.118, 76821 Mont Saint Aignan cedex, France.*

Plant glycoproteins have complex and high mannose type N-linked glycans. In contrast to their mammalian homologues, plant complex N-glycans have a xylose β-1,2 linked to the β-Man of the core and a fucose residue involved in an α-1,3 linkage with the proximal GlcNAc residue of the chitobiose unit. Antibodies specific for these plant-specific structural particularities have been used to determine where β-1,2 xylosylation and α-1,3 fucosylation occur in the Golgi apparatus during the intracellular transport of plant glycoproteins through the secretory pathway.

Many plant vacuolar and secreted proteins have N-linked glycans, but N-glycosylation is not necessary for transport of most glycoproteins from the endoplasmic reticulum to the vacuole. This contrasts with protein secretion which is inhibited when plant cells are treated with tunicamycin. However,

Arabidopsis mutants that are unable to mature complex N-glycans or plant cells treated with inhibitors of glycan processing such as castanospermine or bromoconduritol are not affected in their glycoprotein secretion, while N-glycan processing is effectively blocked by these drugs.

These results show that a very efficient quality control for secreted glycoproteins exists in plants but our results do not favour the involvement of calnexin in this process.

S10. 5.05pm

Glycosylation, Structural Maturation and Trafficking of a Multimeric Vacuolar Protein

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Phaseolin is a homotrimeric vacuolar storage protein. Phaseolin polypeptides contain two potential N-glycosylation sites: Asn252, which is glycosylated with 100% efficiency, and Asn341, which is glycosylated with 50–70% efficiency. We show that glycosylation at Asn341 can occur post-translationally and that its efficiency is enhanced in mutants in which either the first glycosylation site has been inactivated or conformational maturation has been severely affected. Moreover, during passage through the Golgi complex the glycan attached to Asn341 cannot acquire a complex structure and inhibits processing of the glycan attached to Asn252. Finally, we show that glycosylation slows down trimerization, a conformational maturation step that is necessary for the intracellular transport of phaseolin, and partially inhibits transient association between monomers and the binding protein BiP. On the whole, these observations indicate that glycosylation modulates structural maturation of phaseolin in the ER, either directly or by affecting the affinity between molecular chaperones and phaseolin polypeptides.

S10. 5.25pm

In vitro Synthesis of a Microfibrillar (1 → 3)-β-Glucan by *Lolium multiflorum* (1 → 3)-β-Glucan Synthase Enriched by Product Entrapment

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A simple, rapid procedure has been developed to purify a membrane-bound (1 → 3)-β-glucan synthase from *Lolium multiflorum* endosperm cells. After solubilization with CHAPS and incubation with substrate (1 mM UDP-glucose) and effectors (Ca^{2+}, cellobiose), the enzyme was recovered associated with the reaction product by low speed centrifugation (product entrapment). By this procedure, the (1 → 3)-β-glucan synthase was purified 60-fold with respect to the solubilized enzyme preparation. SDS-PAGE analysis of the enriched fraction revealed six major polypeptides (17, 19, 30, 31, 32, and 55 kDa). Comparison of this SDS-PAGE pattern with the polypeptide profile of an immunoprecipitated (1 → 3)-β-glucan synthase preparation suggests that polypeptides at 30–31 and 55–58 kDa are the most likely candidates for participation in (1 → 3)-β-glucan synthesis. Furthermore, in previous work from this laboratory [1], a photoreactive analogue of UNP-glucose (5-[3-(*p*-azidosalicylamide)]allyl-UDP-glucose) specifically labelled at 31 kDa band in an immunoprecipitated synthase preparation, suggesting that this polypeptide bears a UDP-glucose binding site and is involved in *L. multiflorum* (1 → 3)-β-glucan synthase activity.

The product synthesized *in vitro* by the purified (1 → 3)-β-glucan synthase was characterized by methylation analysis, ^{1}H- and ^{13}C-NMR spectroscopy, enzymatic hydrolysis, X-ray diffraction, size exclusion chromatography, transmission electron microscopy and UV-induced fluorescence in the presence of a (1 → 3)-β-glucan specific fluorochrome. The results obtained with these techniques clearly demonstrate that the *in vitro* product is a microfibrillar (1 → 3)-β-glucan of a high DP but of a low crystallinity.

1. Meikle *et al.* (1991) *J Biol Chem* **266**: 22569.

S10. 5.45pm

Molecular Characterization of Arabinogalactan-Proteins (AGPS) from Styles of *Nicotiana Alata*, an Ornamental Tobacco

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Arabinogalactan-proteins (AGPs), a family of proteoglycans, are common components of most plant tissues, plant secretions, and suspension cultured plant cells. They are typically rich in carbohydrate (usually $>90\%$) that contains a high proportion of galactose and arabinose with a structure based on a (1–3)-β-linked galactan backbone branched through C(O)6 to galactosyl sidechains, some of which terminate in arabinofuranosyl residues. The protein is usually a minor component with characteristically high levels of hydroxyproline (Hyp), Ser and Ala. Recently AGPs have been implicated in plant growth and development, as determinants of cellular identity and in the control of somatic embryogenesis. We have cloned and sequenced cDNAs for AGP protein backbones from styles of *Nicotiana alata* [1] and from cell suspension culture of pear [2] and tobacco. Some backbones are consistent with the concept of a 'classical' AGP, a protein consisting mainly of Hyp/Pro, Ala, Ser, Thr residues, but others are different and have an unexpected domain structure. It is likely that these 'non-classical' AGP protein backbones undergo extensive processing resulting in a protein similar to that of the 'classical' AGPs. Systematic characterisation of AGPs will enable us to address some of the issues relating to regulation, processing and function of this family of proteoglycans.

1. Du *et al.* (1994) *The Plant Cell* **6**: 1643–53.
2. Chen *et al.* (1994) *PNAS* **91**: 10305–09; unpublished observations.

S10. 6.00pm

Isolation and Characterization of a Tunicamycin-Resistant Soybean Cell Line

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A tunicamycin-resistant soybean cell line was developed by gradually increasing the concentration of tunicamycin (TM) in the growth medium. At the final stage, the resistant cells could survive in media containing 60 $\mu g\,ml^{-1}$ of TM, whereas normal cells show a greatly retarded growth rate at 0.5 $\mu g\,ml^{-1}$ of antibiotic. The TM-resistant cells had a greater than 40-fold increase in the activity of the enzyme, UDP-GlcNAc:dolichyl-P GlcNAc-1-P transferase (GPT), two- to three-fold increase in the activity of dolichyl-P-mannose synthase, but no increase in the activities of other enzymes of the lipid-linked saccharide pathway such as dolichyl-P-glucose synthase or mannosyl transferases. There was also no change in the activities of the glycoprotein processing enzymes, glucosidase I or glucosidase II as compared to wild type cells. The increase in GPT was due to an increased production of enzyme as seen by a dramatic increase in the amount of a 39 kDa protein, which is presumed to be the GPT protein. The GPT from TM-resistant cells was equally as sensitive to TM as was the wild type enzyme, but was considerably more labile to temperatures above 30°. The GPT activity in TM-resistant-cells was greatly stimulated by exogenous dolichyl-P. The spectrum of oligosaccharides from labelled lipid-linked oligosaccharides was similar in wild type and TM-resistant soybean cells, but the resistant cells had significantly greater amounts of the shorter and much lower amounts of the larger-sized oligosaccharides.

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S10

Monosaccharide and Polysaccharide Changes in Cotton Fibres During Development and Drought Stress

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Monosaccharides and several polysaccharide constituents of the primary cell wall have been determined in cotton fibres during boll development and from plants subjected to varying levels of drought stress. Cotton fibres were sequentially extracted with cold water, 0.5% oxalate, 0.1 N HCl and 10 N NaOH, and the extracts were analysed by HPAEC-PAD. Two polysaccharides had the highest concentration in fibres from control plants with decreasing concentrations in fibres from plants subjected to moderate and high stress. They were almost completely absent in fibres from severe drought stressed plants. Two other polysaccharides were increased in fibres from plants subjected to higher stress. The fibre extracts from drought stressed plants had higher levels of arabinose and ribose and reduced levels of glucose and fructose than the fibre extracts from control plants. This investigation utilized one variety grown in a greenhouse and three other varieties grown under field conditions. With respect to the drought stress of the plants, the polysaccharide differences appear to reflect the status of the plants over a relatively longer period and the monosaccharide differences are indicative of a shorter period. The relative concentrations of three polysaccharides changed during development suggestive of interconversions. Two of the polysaccharides studied during development were chromatographically identical with the two polysaccharides that were increased in higher drought stress. The use of this analysis to determine optimal timing for irrigation is being investigated.

S10 POSTERS

S10

Mode of Action of Two Enzymes That Cleave the Backbone of Rhamnogalacturonan I

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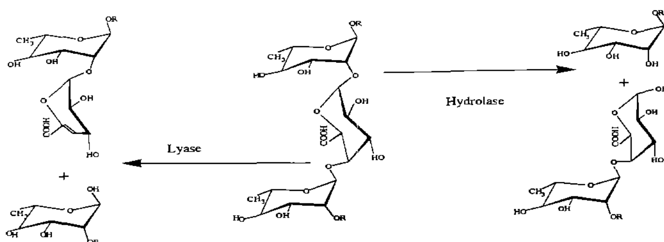
We have investigated the mode of action of two recombinant enzymes [1] that fragment the backbone of the pectic polysaccharide rhamnogalacturonan I. NMR, MS, and UV analyses establish that RGase A is an *endo*hydrolase and RGase B is an *endo*lyase (see below).

Supported by US Dept of Energy DE-FG05-93ER20115.



1. Koford *et al.* (1994) *J Biol Chem* **269**: 29102.

S10

Bioactivity of Animal Oligosaccharides in Plants

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Recently we have demonstrated suppression of fusicoccin (Fc)-induced elongation, rhizogenesis induction, and enhancement of auxin-induced formation of callus and meristematic zones in immature wheat embryo culture by the synthetic pentasaccharide xyloglucan fragment (XG5). As L-Fuc is known to play a crucial role in the biological activity of this class of oligosaccharide elicitors, we have studied another large group of (fucosylated) oligosaccharides from human milk and blood group substances with the different cores (lactose, lactosamine, Galβ1-3GalNAc, lacto-*N*-tetraose, lacto-*N*-*neo*-tetraose, lacto-*N*-hexaose, lacto-*N*-*neo*-hexaose, para-lacto-*N*-hexaose, para-lacto-*N*-*neo*-hexaose, lacto-*N*-octaose). Lacto-*N*-difucohexaose I and Fucα1-2Galβ1-3GalNAc had the same activity as XG5 in tissue culture and the opposite in maize apecal root segment FC-induced elongation. The other oligosaccharides may be sub-divided into two groups. The first group is similar to XG5 in both bioassays, the other one is inactive.

S10

Purification of a Mung Bean α1-3-Fucosyltransferase

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An α1-3-fucosyltransferase which transfers fucose from GDP-fucose into α1-3-linkage to the inner *N*-acetylglucosaminyl residue of an N-glycan was purified from mung bean seedlings by chromatography on DE 52, Affigel Blue, chromatofocusing, gelfiltration and an affinity column to apparent homogeneity with a mobility on SDS-Page of about 65 kDa. The enzyme showed maximal activity at a pH near 7.0 and required 10–15 mM of the divalent cations Mn^{2+} or Zn^{2+}. Triton X-100 was not required, although enhanced activity was found from 0.1–0.5%. The activity was not inhibited by *N*-ethylmaleimide. A substrate specificity for N-glycans with an unsubstituted *N*-acetylglucosaminyl residue at the terminal position linked β1-2 to the α1-3-antennae of the core was found, whereas a fucose α1-6-linked to the inner GlcNAc had no negative effect on the activity. No transfer was detected to *N*-acetyllactosamine or lacto-*N*-biose.

The enzyme preparation makes it possible to produce α1-3-monofucosylated and – with an appropriate acceptor substrate – α1-3/α1-6-difucosylated structures which can be used for further investigations on the biosynthetic pathway of glycans in different organisms and for immunological studies.

S10

The Structure of Xyloglucans from Solanaceous Plants

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The xyloglucans (XGs) secreted by suspension cultured tobacco and tomato cells were shown, by spectroscopic analyses of their oligosaccharide subunits, to have different structures. The XGs from both species are typical in that they are composed of a (1 → 4)-linked β-D-Glc*p* backbone substituted at C-6 with various side chains. The XGs from both species have α-D-Xyl*p* and α-L-Ara*f*-(1 → 2)-α-D-Xyl*p* side chains. However, the tomato XG was significantly more complex, having β-D-Gal*p*-(1 → 2)-α-D-Xyl*p* and β-Ara*f*-(1 → 3)-α-L-Ara*f*-(1 → 2)-α-D-Xyl*p* side chains that are not present in the tobacco XG. Many of the β-D-Glc*p* residues in the backbone of both XGs bear an *O*-acetyl substituent at C-6 rather than a glycosyl side chain. We hypothesize that the combined *O*-acetyl and glycosidic substitution patterns of XGs maintain a molecular topology that leads to proper assembly of the XG-cellulose network in cell walls.

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S11. GOLGI ENZYME TARGETING

Chairs: Stuart Kornfeld, Carlos Hirschberg

S11. 4.00pm

Introduction

S11. 4.05pm

Targeting of Glycosyltransferases to the Golgi Apparatus

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The Golgi apparatus maintains a highly organized structure in spite of intense membrane traffic which flows into and out of this organelle. Resident Golgi proteins must have localization signals to ensure that they are targeted to the correct Golgi compartment. There are a number of distinct groups of Golgi membrane proteins, including glycosyltransferases, recycling *trans*-Golgi network proteins, peripheral membrane proteins, receptors and viral glycoproteins. Recent studies have indicated that there are a number of different mechanisms for retaining proteins to the Golgi apparatus.

We have investigated the mechanism for the localization of two resident Golgi glycosyltransferases, namely β1,4 galactosyltransferase (Gal T) and *N*-acetylglucosaminyltransferase I (GlcNAc TI). These membrane enzymes reside in the *trans* and *medial* Golgi compartments, respectively. Like other Golgi glycosyltransferases, Gal T and GlcNAc TI are membrane bound proteins with an N_{in}/C_{out} orientation, containing a short cytoplasmic domain, a dual signal/anchor domain, and a lumenal catalytic domain. Our initial studies showed that the transmembrane domain of both glycosyltransferases can target hybrid molecules to the correct Golgi cisternae. We have further examined the contribution of each of the three domains of GlcNAc TI in *medial*-Golgi localization by analysing the localization of hybrid molecules stably expressed in mouse cells and have shown that the protein sequences from all three domains of GlcNAc TI are necessary for complete Golgi retention.

Based on post-translational modifications which probably occur in the *trans*-Golgi network we have demonstrated that Golgi localization of Gal T primarily involves active retention and not retrieval. A variety of approaches including cross-linking and immunoprecipitation experiments indicate that these glycosyltransferases exist as aggregates within the Golgi apparatus. This ability to aggregate may be a requirement for Golgi retention.

S11. 4.35pm

Signals and Mechanisms for the Golgi Retention of the α2,6-Sialyltransferase

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The α2,6-sialyltransferase (ST) is a terminal glycosyltransferase localized in the trans Golgi and trans Golgi network. Immunofluorescence microscopic localization of ST mutants and chimeras suggests that cytoplasmic and lumenal lysine residues flanking the ST transmembrane region are particularly important for efficient Golgi retention, while the specific amino acid composition and the length of the transmembrane region is not crucial. Inefficient Golgi retention of several chimeras possessing exogenous lumenal sequences also suggests that this region plays a role in the correct folding of more amino terminal Golgi retention sequences and points to the conformation dependence of these retention sequences. We have begun to test the possibility that the ST forms insoluble oligomers in the specific environment of the late Golgi, preventing transport out of the Golgi, and leading to enzyme retention. Analysis of rat liver Golgi and ER membranes demonstrated that ~35% of the ST protein forms a disulfide-bonded dimer in the Golgi. Interestingly, the dimer is catalytically inactive due to a weak affinity for its donor molecule, CMP-sialic acid. However, the ST dimer is able to bind galactose-terminated substrates and may function as a galactose-specific lectin in the Golgi. While only monomer and dimer forms of the ST were detected following glycerol gradient sedimentation analysis, we found that the dimer form of the ST preferentially becomes insoluble at pH 6.4, the pH of the late Golgi. These results suggest the possibility that the ST dimer initiates or augments the formation of weakly associated ST oligomers leading to this enzyme's retention in the Golgi.

S11. 5.05pm

Cloning of the Golgi UDP-GlcNAc Transporter of *Kluyveromyces lactis*

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The outer-chain carbohydrate mannoproteins of *K. lactis* differ from those of *S. cerevisiae* in that they contain a terminal αGlcNAc. Douglas and Ballou [1] described a mutant of *K. lactis* (mnn2-2) which lacks the terminal αGlcNAc. Detailed biochemical studies by these authors ruled out the possibility that the defect was the result of a lack of the corresponding *N*-acetylglucosaminyltransferase, endogenous acceptors, or impaired biosynthesis of UDP-GlcNAc. We have determined that this mutant phenotype is the result of a defect in transport of EDP-GlcNAc into Golgi vesicles. This was demonstrated by preparing vesicles from wild-type and mnn2-2 cells and showing that both vesicles were able to translocate into their lumen GDP-mannose while only those from wild-type cells were also able to transport UDP-GlcNAc.

The mutant cell line was then transformed with a wild-type *K. lactis* genomic library. Transformants that recovered the wild-type phenotype were selected using FITC-GS II lectin and a fluorescent activated cell sorter. A 2.6 kb DNA fragment was sequenced and found to code for a hydrophobic protein which appears to have multiple transmembrane domains and presumably is the UDP-GlcNAc transporter.

1. Douglas, Ballou (1982) *Biochemistry*, **21**: 1561.

S11. 5.35pm

Medial-Golgi Localization and an Ecto-type Form of UDP-*N*-acetyl-glucosamine: β-D-mannoside β-1,4*N*-acetylglucosaminyltransferase III

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UDP-*N*-acetylglucosamine:β-D-mannosideβ-1,4*N*-acetylglucosaminyltransferase III (GnT-III; EC 2.4.1.144) is a key enzyme in the regulation of branch formation in N-glycans. A GnT-III-specific polyclonal antibody immunoprecipitated GnT-III activity with molecular masses of 88 kDa and 74 kDa from mouse melanoma B16-F1 cells transfected with the rat *GnT-III* gene. Immunocytochemical studies showed that the immunoreactive GnT-III was localized mainly in the medial-Golgi region and partially in the cytosol and plasma membrane. To compare artificial expression of GnT-III with natural expression, GnT-III expression in normal rat kidney was investigated immunohistochemically. GnT-III was mainly found in the apical cytoplasm and plasma membrane of the brush border rather than in the Golgi apparatus of proximal tubular cells. The immunoreactive GnT-III in the brush border membranes had molecular masses of 38–44 kDa and had lower GnT-III activity than the Golgi form. These results provide evidence for the presence of an 'ecto-GnT-III' in the plasma membrane of proximal tubular cells of the kidney. These observations also suggest that the sorting and translocation of GnT-III are controlled not only by the GnT-III molecule itself but also by the cell type-specific sorting environment.

S11. 5.55pm

The Stem Region of the Polypeptide:αGalNAc Transferase Contains a *cis* Golgi Retention Signal

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The polypeptide αGalNAc transferase catalyses the first step of O-glycosylation. This enzyme as well as its reaction products have recently been localized in the *cis* Golgi stacks and may therefore be considered a specific marker for the *cis* Golgi compartment. The mechanism and sequences responsible for the retention of this enzyme in the *cis* Golgi are the object of our investigation.

cDNA encoding the bovine αGalNAc transferase and containing a tag sequence at the 3′ end was expressed in different cell lines and the product was localized by confocal immunofluorescence microscopy using an antibody directed at the tag sequence. In COS-7, HeLa, HOS and MCF7 cell lines transfected with the construct encoding the full-length sequence of the enzyme, the transferase was found exclusively in an intracellular compartment which is likely to be the *cis* Golgi. The cellular localization of the enzyme was not altered by deletion of its cytoplasmic tail or substitution of some of the aminoacids in the transmembrane domain of the molecule. However, deletion of 60 aminoacids situated on the luminal side of the enzyme near the transmembrane domain drastically

changed the intracellular localization of the transferase. These results indicate that, in contrast to glycosyltransferases located in the median or *trans* Golgi, the stem region – and not the cytoplasmic tail or the transmembrane domain – is responsible for the retention of the αGalNAc transferase in the *cis* Golgi compartment.

S11 POSTERS

S11

Characterization of Man_9-mannosidase from Human Kidney Expressed in COS 1 Cells

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Man_9-mannosidase cloned from a human kidney cDNA library [1] was transiently expressed in COS 1 cells, resulting in a more than 20-fold increase of a 1,2-specific mannosidase activity (as determined with (^{14}C)$Man_9GlcNAc_2$). The expressed activity was strongly inhibited by 1-deoxymannojirimycin (50% at 100 μM). The protein has a molecular mass of 73 kDa and was found to be glycosylated at one of three potential N-glycosylation sites. Approximately 50% of the N-linked oligosaccharide chains are cleaved by endo H. Complete susceptibility to endo H was obtained when transfected cells were cultured in the presence of the Golgi mannosidase II inhibitor swainsonine indicating partial processing by medial or trans Golgi resident enzymes. This observation is consistent with the results of indirect immunofluorescence studies, pointing to a localization of the expressed Man_9-mannosidase protein predominantly in a juxtanuclear Golgi region. The Golgi localization differs from that one determined recently for pig liver Man_9-mannosidase which was immunolocalized in the ER and transient vesicles. Proteolytic experiments indicate that both the human kidney and pig liver enzyme are type II transmembrane proteins.

1. Bause E *et al.* (1993) *Eur J Biochem* **217**: 533–40.
2. Roth J *et al.* (1990) *Eur J Cell Biol* **53**: 131–41.

S11

Specific Detection of Transfected α1,3Fucosyltransferase (Fuc-T 5) in the Golgi Apparatus by Immunocytochemistry

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The fucosyltransferases (Fuc-Ts) constitute a family of transferases incorporating terminal fucose residues into glycoprotein or glycolipid glycans. Their putative cellular location is the Golgi apparatus. Immunocytochemical localization has been hampered by the lack of specific antibodies due to extensive homologies of Fuc-T III, V and VI at the protein level. Here we report the first immunocytochemical detection of a fucosyltransferase, in particular Fuc-T V. Plamids encoding Fuc-T V were transfected into COS-cells and enzyme expression detected by using an antibody raised against a specific peptide stretch of Fuc-T V which is absent in the closely related Fuc-T III and Fuc-T VI. This antiserum was shown not to crossreact with the homologous Fuc-Ts transfected into COS-cells and tested for the presence of corresponding activities in COS cell lysates. Another specific antiserum to Fuc-T V was elicited by expressing the N-terminal part of the coding sequence as a fusion protein with β-galactosidase in *E. coli.* Both antisera were characterized by ELISA, immunofluorescence and immunoprecipitation of Fuc-T V metabolically labelled in transiently transfected COS-cells.

These antisera will be used as tools to study developmental and spatial regulation of expression of Fuc-T V.

Supported by the SNSF.

S11

Targeting of Transfected β1,4Galactosyltransferase to the Golgi Apparatus and the Cell Surface in COS-1 cells: Role of the N-terminal 13 Amino Acids

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Earlier work has demonstrated that precursors of β1,4galactosyltransferase (GT) exist as 42 kDa (GT_s) and 44 kDa (GT_l) forms corresponding to two initiation *met* sites separated by 13 amino acids. Data reported by other groups on differential targeting of these forms are conflicting. Here we show by immunofluorescence using monoclonal or protein-specific polyclonal antibodies that COS-1 cells, transiently transfected with a plasmid encoding GT_l forms under the control of the CMV promoter, expressed the recombinant enzyme both in the Golgi apparatus and on the cell surface. Conversely, expression of GT_s was restricted to the Golgi apparatus. Cell surface appearance of GT_l occurred in two aspects: as evenly distributed ectoenzyme on attached cells and in a condensed form on rounded (dead) cells suggesting toxic effects of overexpression on the cell surface. Transfection of the same plasmids into CHO cells which lack a replicating system did not lead to cell surface expression of GT suggesting that in order for the N-terminus to specify for cell surface expression a high level of expression is also required.

Supported by SNSF.

S11

Purification and Characterization of Human Lymphoid *N*-Acetylglucosamine-1-phosphodiester α-*N*-acetylglucosaminidase

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The intracellular segregation of many lysosomal enzymes to lysosomes is dependent on those enzymes possessing a targeting signal, mannose-6-phosphate, on their oligosaccharide side chains. This marker is generated through the concerted action of two key enzymes: (1) UDP-GlcNAc:glycoprotein GlcNAc-1-phosphotransferase (GlcNAcPTase); and (2) the *N*-acetylglucosamine-1-phosphodiester α-*N*-acetylglucosaminidase (uncovering enzyme). Although the uncovering enzyme plays a key role in the biosynthesis and targeting of lysosomal enzymes to lysosomes, little information is currently available concerning its structure, intracellular location and the gene products that control its expression. Preliminary studies in our laboratory using cultured human lymphoid cells from a healthy individual produced a partially purified enzyme [1]. The current results demonstrate that the uncovering enzyme isolated from human lymphoid cells can be purified through a sequence of separate

chromatographic methodologies including Lentil Lectin Sepharose, DEAE-Sephacel, Affigel 501 and Zn-(II)-IDA Sepharose followed by separation elution of polypeptide(s) on preparative SDS-gel electrophoresis. Analytical SDS-PAGE of the eluted and separated proteins followed by activity measurements revealed that the uncovering enzyme migrated as a doublet of apparent molecular weights of 106 kDa and 102 kDa. Characterization studies including pH optimum and kinetic properties with respect to different substrates were also carried out using the purified protein.

Supported by NIH grant NS12138 to Arnold L. Miller, PhD.

1. Zhao K, Miller AL (1993) In: *'Molecular Mechanisms of Membrane Traffic'* (Moore DJ, Bergeron JJM, Howell KM, eds) New York: Springer-Verlag. Vol. H74:371–72.

S11

Release of Sialyltransferases from Golgi membranes in Liver by a Cathepsin D-like proteinase

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Previous work showed that Galβ1-4GlcNAcα2-6sialyltransferase (2-6NST) was released from the lumenal face of the Golgi by the action of endogenous cathepsin D which was believed to be the lysosomal enzyme [1]. The release of sialyltransferase was particularly prominent during the acute phase response to inflammation. We have now shown that two other sialyltransferases are released by a similar mechanism. These are Galβ1-3(4)GlcNAcα2-3sialyltransferase (2-3NST), and SAT-1 (which adds NeuAc to form NeuAcα2-3Galβ1-4GlcCeramide). There was a three-fold increase in the levels of Golgi 2-6NST in inflammation, but the other sialyltransferases decreased by about 50%. However, all three enzymes were released from the Golgi by incubation at reduced pH which activates the endogenous cathepsin D. Pepstatin A was found to block the release of all three enzymes providing support for the idea that cathepsin D is the proteinase that clips the catalytic portion of the sialyltransferases from their stem and anchor regions. A similar conclusion was made following the use of liver slices to follow the release of sialyltransferases from liver after inflammation.

1. Lammers G, Jamieson JC (1988) *Biochem J* **256**: 623–31.

S11

Morphological Changes in the Hepatocyte Golgi Apparatus which Accompany the Release of α2-6Sialyltransferase during the Acute Phase Response to Inflammation

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Following turpentine-injection (TI) to induce the acute phase response to inflammation, the TGN-resident Galβ1-4GlcNAcα2-6sialyltransferase (2,6ST) is released into the serum with a time-curve (initial increase: 8–12 h, max: 48 h) which parallels that of acute phase reactants. To obtain a morphological correlate of this event, the Golgi region of hepatocytes was analysed with transmission electron microscopy 1–66 h following TI. Changes occurred both in the types of materials processed by the Golgi and in the morphology of the Golgi membranes. Lipoprotein particles were processed at 1, 2, 6, 12 and 16 h, and again at 66 h post-TI; a granulofilamentous material was processed at 12, 16, 24 and 48 h post-TI. The processing of two visible secretory materials in consecutive, but overlapping time-intervals allowed us to: 1) identify subcomponents of the Golgi and vesicle-populations involved in the transport of the two materials; and 2) to study their sorting. Major changes in Golgi membrane morphology, at the height of 2,6ST release, included: dilation of the mid-saccules, deposition of an electron-dense matrix near the TGN and the acquisition of lysosomal cytidine monophosphatase reactivity by the TGN.

S12. GLYCOBIOLOGY OF YEASTS, MOLDS AND BACTERIA

Chairs: Howard Bussey, Phil Robbins

S12. 4.00pm

Molecular Mimicry and Structural Variations in Lipopolysaccharides of the Ulcer Associated Bacterium, *Helicobacter pylori*

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Characterizations of lipopolysaccharides (LPS) were undertaken to provide a molecular basis for strain to strain antigenic variations and interactions of bacterial cell surface components with gastric mucosa. Samples from rough- and smooth-forms of the *H. pylori* type strain [NCTC 11637; ATCC 43504]; P466 [a poorly haemagglutinating strain binding to Le^b antigen] and MO19 [a haemagglutinating strain not binding to epithelial cell]; and strain C1 [serogroup O:3; from J.L. Penner] were used for studies of LPS and products from chemical and enzymic degradation, by chemical analyses, nuclear magnetic resonance spectroscopy, and mass spectrometry [fast atom bombardment and electron impact]. The primary structure was assigned for the O-chain, an intervening region, and the core OS of the type strain. The discovery of repeating O chains of fucosylated *N*-acetyllactosaminoglycans with Le^x determinants provided a remarkable example of bacterial molecular mimicry of human cell surfaces. The inner core OS region was established as a hexasaccharide unit, with an attached phosphoric monoester and a reducing 3-deoxy-D-*manno*-octulosonic acid terminus, and was identical in the four LPS samples. LPS from the three other strains differed from that of the type strain in one or more of: elongation of O chains; termination of O chain by the Le^y determinant; or the sugar units in the intervening

region including those of the rare sugar, D-*glycero*-D-*manno*-heptose.

The mimicry of Lewis blood group antigens on the surface of *H. pylori* strains may have important implications for colonization and pathogenesis, especially in the expression of tumour-associated carbohydrate antigens on the bacterial surface in relation to carcinogenesis and immune suppression. The results may also aid the development of specific antibodies for diagnosis of infections.

S12. 4.20pm

Biosynthesis of the *E. coli* K4 Capsule Polysaccharide – a Parallel System for Studies of Glycosyltransferases in Chondroitin Formation

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The *E. coli* strain K4 synthesizes a capsule polysaccharide [GalNAc-GlcA(Fru)]$_n$ with the carbohydrate backbone identical to chondroitin [1]. The branching fructose units can easily be removed from the polymer by mild acidic hydrolysis. In order to assay the GlcA and GalNAc transferase activities of the bacterial membrane, acceptor oligosaccharide substrates derived from the K4 polysaccharide were prepared using both enzymatic degradation and partial hydrazinolysis followed by deamination with nitrous acid. Bacterial membranes were assayed for glycosyltransferase activities using these acceptor oligosaccharides and radiolabelled UDP-[^{14}C]GlcA resp UDP-[^{3}H]GalNAc. It was found that defructosylated (chondroitin) oligosaccharides [GalNAc-GlcA]$_n$-GalNAc could serve as substrates for the GlcA transferase and the GlcA-[GalNAc-GlcA]$_n$-aTal$_R$ were acceptors for the GalNAc transferase. The enzymatically labelled oligosaccharide products were completely degraded with Chondroitinase AC and the [^{14}C]GlcA unit was removed by β-D-glucuronidase. A fructosylated oligosaccharide acceptor [GalNAc-GlcA(Fru)]$_n$ tested for transfer of [^{14}C]GlcA was inactive. These results indicated that the chain elongation reaction of the K4 polysaccharide proceeds by addition of the sugar units one by one from the corresponding UDP-sugars to the nonreducing end of the polymer and before the addition of the fructose units. This mechanism is similar to polymerization of the chondroitin sulfate and makes the biosynthesis of the K4 polysaccharide to an interesting parallel system for studies of the chondroitin sulfate biosynthesis.

1. Rodriguez M-L, Jann B, Jann K (1988) *Eur J Biochem* **177**: 117–24.

S12. 4.40pm

Cloning and Expression of the β-*N*-Acetylglucosaminidase Gene from *Streptococcus pneumoniae*: Generation of Truncated Enzymes with Modified Aglycon Specificity

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The aglycon specificity exhibited by β-*N*-acetylglucosaminidase from *Streptococcus pneumoniae* not only makes it a useful tool for oligosaccharide sequencing, it also makes it a suitable candidate for the investigation of the molecular requirements that dictate substrate/enzyme interactions. We have obtained the gene encoding a β-*N*-acetylglucosaminidase from *S. pneumoniae* by screening an expression library for arylglycoside hydrolytic activity. Clones of different nucleotide sizes were obtained and DNA sequencing revealed a gene of 3933 bp. Analysis of the translated protein of 1311 amino acids (144 210 Da) identified a tandem repeat within which lies a sequence homologous with six other hexosaminidase gene products from a wide variety of species. Also found were an N-terminal putative secretion signal peptide and a C-terminal cell sorting/anchorage motif also found in over 20 other gram positive surface proteins. The expression of an almost complete DNA clone in *E. coli* produced a functional and authentic β-*N*-acetylglucosaminidase with aglycon specificity identical to the wild type enzyme. However, enzymes produced from truncated DNA clones show more restricted aglycon specificity and were unable to hydrolyse terminal β1-2GlcNAc residues from N-glycans containing a bisecting *N*-acetylglucosamine. The availability of truncated enzymes that have altered substrate specificities allows structural analyses to be made of catalytic and oligosaccharide recognition protein domains that enhance functional activity.

S12. 5.00pm

The Essential Role and Complexity of GPI Biosynthesis in Yeasts

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The *Saccharomyces cerevisiae* GPI anchoring mutants *gpi1*, *gpi2*, and *gpi3*, isolated by screening for strains blocked in [^{3}H]inositol incorporation into protein, are defective *in vitro* in the synthesis of GlcNAc-PI, the first step in GPI assembly. Single *gpi* mutants are temperature-sensitive and double *gpi* mutants are inviable, indicating that GPI synthesis is required for growth of *S. cerevisiae*. The *GPI1* and *GPI2* genes were cloned by complementation, and encode hydrophobic proteins that do not resemble other proteins known to participate in GPI assembly. *GPI3* encodes a protein similar to the human PIG-A protein, which also participates in GlcNAc-PI synthesis. Overexpression of the Gpi2 protein gives partial suppression of the *gpi1* mutant's temperature sensitivity suggesting that the Gpi1 and Gpi2 proteins interact with one another *in vivo*. Taken together, our results with *S. cerevisiae gpi* mutants, and others' results with human genes required for GlcNAc-PI synthesis, indicate that at least four proteins participate in the first step in GPI synthesis. We have cloned the *Schizosaccharomyces pombe GPI1* gene by complementing the *S. cerevisiae gpi1* mutant with an *S. pombe* cDNA library. Disruption of the *S. pombe GPI1* gene is lethal, confirming the importance of GPIs in unicellular eukaryotes. Using PCR amplification, we have identified a *GPI1* gene in the filamentous fungus *Aspergillus nidulans*. We are exploring the effects of GPI anchoring mutations on fungal growth and morphogenesis.

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S12. 5.20pm

MNN4, Gene for the Mannosylphosphate Addition of N-Glycosylation in *Saccharomyces cerevisiae*

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The *mnn4* mutant contains a low phosphate in the outer chain of N-linked oligosaccharides. Although the mutant shows a dominant phenotype, the dominancy is suppressed by osmotic stabilizers like KCl or sorbitol. This mutation is mapped on the left arm of chromosome XI near the *TRP3* and *URA3* loci. To analyse the effect of this mutation on the mannosylphosphate addition in the core oligosaccharide formed in the ER, the cell adsorption to the anion exchange QAE-beads was compared between Δ*och1mnn1* double and Δ*och1mnn1mnn4* triple mutants, both of which lack a mannose outer chain due to the lack of initiation specific α-1,6-mannosyltransferase encoded by *OCH1* gene. The latter cells were adsorpted to the beads significantly less than the former cells, suggesting that the *mnn4* mutation may affect the mannosylphosphate addition not only to the outer chain but also to the ER core portion.

To clone the wild type *MNN4* gene, the recessive conditions were established that distinguishes Alcian Blue dye binding of cells between *mnn1* and *mnn1mnn4* strains. One of the DNA fragments which were expected to include the *MNN4* loci (from ATCC) was able to complement the *mnn4* mutation. According to the sequence data of Chr. XI, the minimum region of 11.7 kb contained two intact ORFs, but neither was able to complement the *mnn4* mutation. Since these two ORFs were closely linked each other in the same ORF, the gap region was sequenced, revealing a new big ORF of 3534 bp after nine single base corrections. The predicted protein had a membrane spanning region near the N-terminus, suggesting that the *MNN4* may encode a gene for mannosylphosphate transferase, which is responsible for the oligosaccharide phosphorylation in *S. cerevisiae*.

S12. 5.40pm

Localization of the Kre2p/Mnt1p α1,2-mannosyltransferase to the *Saccharomyces cerevisiae* Golgi Complex

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The yeast Kre2p/Mnt1p α1,2-mannosyltransferase is a type II membrane protein with a short cytoplasmic amino-terminus, a membrane-spanning region, and a large catalytic lumenal domain containing one N-glycosylation site. Kre2p/Mnt1p adds the third mannose residue to O-linked mannosyl side-chains of proteins and is also involved in N-linked outer chain elaboration. Anti-Kre2p/Mnt1p antibodies identify a 60-kD integral membrane protein which is progressively N-glycosylated in an *MNN1*-dependent manner. Kre2p/Mnt1p is localized in a Golgi compartment which overlaps with that containing the medial Golgi mannosyltransferase, Mnn1p, and distinct from that comprising the late Golgi protein, Kex1p. To determine which regions of Kre2p/Mnt1p were required for Golgi localization, Kre2p/Mnt1p mutant proteins were assembled by substitution of Kre2p domains with equivalent sequences from the vacuolar proteins DPAP B and Pho8p. All chimeric proteins were tested for correct topology, *in vitro* and *in vivo* activity, and were localized intracellularly by indirect immunofluorescence. The results demonstrate that the short N-terminal cytoplasmic tail domain is necessary for correct Kre2p Golgi localization, whereas the membrane spanning and stem domains are not. However, a reporter protein can only be localized to the Golgi complex by a region of Kre2p which encompasses the cytoplasmic tail, the TMD, and a partial stem region.

S12. 6.00pm

Synthesis of 'Nod'-Like Chitin Oligosaccharides by the *Xenopus* Developmental Protein DG42

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The *Xenopus DG42* gene is expressed only between the late midblastula and neurulation stages of embryonic development. Recent data base searches show that *DG42* has striking sequence similarity to the *Rhizobium* NodC protein. NodC catalyses the synthesis of chitin oligosaccharides which subsequently are transformed into bacterium-plant root signalling molecules. We find that the DG42 protein made in an *in vitro* coupled transcription-translation system catalyses the synthesis of an array of chitin oligosaccharides. The result suggests the intriguing possibility that a bacterium-plant type of 'nod' signalling system may operate during early stages of vertebrate embryonic development and raises issues about the use of chitin synthase inhibitors as fungal-specific drugs.

S12 POSTERS

S12

Functional Domains of Bovine β-1,4 Galactosyltransferase

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β-1,4 Galactosyltransferase (β-1,4 GT) is a type II membrane protein, composed of a short amino-terminal cytoplasmic tail, a signal anchor domain, a stem region and a domain that catalyses the transfer of galactose from UDP-galactose to the terminal *N*-acetylglucosamine (NAG) residue in glycoproteins and glycolipids. Site-directed mutagenesis was used to identify in bovine β-1,4 GT the regions that interact with *N*-acetylglucosamine and UDP-galactose. A series of N-terminal deletion mutants were constructed by polymerase chain reaction, and expressed in *E. coli*. Both fusion and non-fusion recombinant proteins localized in inclusion bodies, as insoluble aggregates, and required 'oxido-shuffling' reagents for the regeneration of the enzyme activity. Decreasing the length of the N-terminal region of β-1,4 GT affects the solubility of the enzyme which is restored with increasing concentrations of NaCl. Enzyme kinetic analysis for mutants lacking residues 1–129 revealed that the K_m values for NAG, chitobiose and chitotriose were not significantly different from bovine milk β-1,4 GT. Two GT fragments: GT-d129NAG, (residues 130–257), and GT-d257UDP (residues 258 to end) bound UDP- and NAG agarose column in the presence of 25 mM $MnCl^{+2}$. Elution of GT-d257UDP from UDP-galactose and NAG agarose columns is dependent on the presence of 50 mM EDTA and

15 mM NAG, while GT-d129NAG can be eluted with 15 mM NAG in the absence of 50 mM EDTA, suggesting that the amino-terminal region binds poorly to Mn^{+} while the carboxy-terminal region of β-1,4 GT binds Mn^{2+} tightly.

S12

Engineering of the Carbohydrate Moiety of Fungal Glycoproteins to a Mammalian Type

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Mammalian-like hybrid oligosaccharides were synthesized on glycoproteins from the filamentous fungus *Trichoderma reesei* by *in vitro* enzymatic modification. In addition, we showed that preincubation of the fungal protein with *Aspergillus saitoi* α1,2 mannosidase improved formation of the hybrid structure. However, it did not appear possible to convert all fungal oligosaccharides to the acceptor substrate for *N*-acetyl glucosaminyl transferase I. During fermentation in different growth media, oligosaccharides are synthesized that predominantly range from Man5Gn2 to Man9Gn2. A small fraction of the N-glycans are larger than Man9Gn2. The presence of small N-glycans seems not to be due to trimming in the extracellular medium. These results indicate that in *Trichoderma reesei*, processing of the oligosaccharides partly resembles that of mammalian cells and partly that of yeast: the majority of the precursor oligosaccharides, that are transferred to the nascent protein chain are trimmed to mannose structures containing nine to five mannose residues. This resembles the first mammalian processing steps. Such trimming steps are not at all observed in *Saccharomyces cerevisiae*. On the other hand, a fraction of the fungal oligosaccharides are extended through addition of mannose residues in linkages different from the α1,2 linkage. This resembles yeast processing.

S12

Heterogeneity of Cell Wall Glycolipids Paralleled with Virulence in *Myocobacterium tuberculosis* Human Isolates

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Tuberculosis is a chronic disease caused by *Mycobacterium tuberculosis* infection. The major pathological changes are immunologically hypersensitive granuloma formation due to the local proliferation or infiltration of immune cells. However, the mechanism for the development of the disease has not been fully understood. The first step of infection is intracellular survival in the phagocytic cells and this process has been reported to be regulated by cell surface glycolipid virulence factors. Since recently, genetical heterogeneity of *M. tuberculosis* among strains has been reported based on DNA fragmentation pattern, we have examined the distribution of cell surface glycolipids (cord factor, sulfolipids, acylated trehaloses and phenolglycolipids) among the virulent (H37Rv, Aoyama B) and avirulent (H37Ra) strains. The glycolipid components were separated by two dimensional thin-layer chromatography of silicagel. Each glycolipid was identified by FAB/MS analysis of intact lipid and GC/MS analysis of fatty acids or carbohydrate moiety. As a result of this, we found a very heterogeneous distribution of glycolipids among strains. Cord factor existed ubiquitously, but sulfolipids did not. Major acylated trehaloses were detected only in virulent strains. Only limited strains produced phenolglycolipids. The results shows the existence of these toxic glycolipids profoundly contributes to the virulence of *M. tuberculosis*. The combined effect and role of each glycolipid will be discussed.

S12

Isolation and Characterization of CMP-NANA Synthase from *Haemophilus ducreyi*

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Haemophilus ducreyi is a gram-negative bacterium that infects the genital epithelium causing the sexually transmitted disease 'chancroid'. Chancroid has been shown to be an independent risk factor for the transmission of AIDS. The outer-membrane lipooligosaccharides (LOS) of *H. ducreyi* have been implicated in the pathology of this disease. Previously, we have reported the presence of terminal lactosamine in the majority of *H. ducreyi* LOS, which can then undergo the addition of sialic acid (*N*-acetyl-neuraminic acid, or NANA) to form terminal sialyllactosamine. Sialic acid is thought to be an important virulence factor and, therefore, we have begun to identify and characterize the enzymes required for the biosynthesis of these sialylated LOS glycoforms in pathogenic strains of *H. ducreyi*.

Although our initial attempts to isolate the sialyltransferase were unsuccessful, we have isolated and characterized the CMP-NANA synthase in the soluble portion of disrupted *H. ducreyi* cells. This enzyme has been purified to homogeneity using a series of dye, hydrophobic interaction, anion exchange, and size-exclusion chromatography steps. We have demonstrated in an *in vitro* assay that this protein is capable of synthesizing CMP-sialic acid in the presence of CTP and sialic acid. Preliminary characterization studies have allowed us to determine the K_m for both substrates, i.e. $\approx$0.7 mM for sialic acid and $\approx$0.1–0.2 mM for CTP, as well the enzymes requirement for Mg^{+2} (activity *vs.* [Mg^{+2}]). N-terminal protein sequence data have also been obtained that show this enzyme to share sequence identity with the other two members of this enzyme family, the CMP-NANA synthase from *E. coli* (40%) and *N. meningitidis* (48%).

S12

A Simple and Rapid Dot Immunoassay for the Serodiagnosis of Tuberculosis using Cord Factor (Trehalose 6,6′-dimycolate) as Antigen

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It is essential in the diagnosis of pulmonary tuberculosis, to demonstrate the tubercule bacilli in the sputum of patients either by smear staining or by isolation. Such conventional methods, however, have many drawbacks, i.e. the detection rate is low in the smear test and isolation by culture requires 4–6 weeks. Therefore, there is a need for the development of a

rapid and reliable diagnostic method (serologic diagnosis) for tuberculosis. In the previous paper,we reported that antibodies against cord factor (trehalose 6,6'-dimycolate), a characteristic cell-wall component of tuberculous bacilli are produced in the serum of patients with pulmonary tuberculosis, and that the detection of anti cord factor antibody is useful in rapid serodiagnosis of tuberculosis. However, microplate ELISA requires a plate reader system and substantial amount of antigenic cord factor. We report here that a simple dot enzyme immunoassay based on the recognition of serum IgG antibody using polyvinyliden difuluoride (PVDF) membrane promising, and that the previously described favourable test characteristics of plate ELISA confirmed. For patients with active and untreated pulmonary tuberculosis, the plate ELISA test had a sensitivity of 81% and a specificity of 96% and the dot ELISA also showed similar levels, showing usefulness of the serodiagnosis of tuberculosis. Dot blot enzyme immunoassay may be the most promising for use as a screening test in situations of restricted technical facilities.

S12

Granuloma Formation by Novel Mycoloyl Glycolipids Paralleled with Cytokine Induction in Mice

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Granulomatous inflammation is the most characteristic change in tuberculosis infection. This is noted by the local proliferation or infiltration of immune cells such as monocytes, mature or immature macrophages or lymphocytes in the lungs, spleen or liver. Tuberculous granulomas are produced by complicated immunological processes. However, the mechanisms for such pathological changes are not well understood. Cord factor (a mycoloyl glycolipid) is an adjuvant-active glycolipid with which granuloma (foreign body type) can be generated without any protein antigen. We have investigated the granuloma forming activity of various mycoloyl glycolipids isolated from *mycobacteria, nocardia,* and *rhodococci*, paralleled with cytokine (TNFa) inducing activity. In this paper, we report that various mycoloyl glycolipids were isolated from *Rhodococcus terrae*, including trehalos, glucose, mannose or fructose esters of mycolate. A newly isolated novel glycolipid 'trehalose trimycolate' showed the highest activity for granuloma formation in mice after intravenous administration of water in oil in water emulsion, trehalose dimycolate, monoacyltrehalose dimycolate and glucose monomycolate also showed significant activity. In contrast, mannose or fructose esters did not show any activity. The importance of carbohydrate moiety and mycoloyl residues to induce granulomas in mice will be stressed.

S12

Analysis of Yeast Mannosylphosphate Transferase

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N-linked oligosaccharides of *Saccharomyces cerevisiae* contain acidic oligosaccharides in which mannosylphosphate residues are attached to the neutral oligosaccharides at both the mannose outer chain and ER core portions. Recently it was found that the *S. cerevisiae* Δ*och1 mnn1* mutant, which produces the ER core form Man8GlcNAc2 lacking a mannose outer chain as a neutral oligosaccharide, still contains acidic oligosaccharides, in which mannosylphosphate is attached to the neutral Man-8GlcNAc2 at the same position as *N*-acetylglucosaminylphosphate is attached to Man8GlcNAc2 in mammalian cells [1]. This result suggests that *S. cerevisiae* may have mannosylphosphate transferases that show a similar substrate specificity to mammalian *N*-acetylglucosaminylphosphate transferases. We analysed these mannosylphosphate transferase activities using membrane fraction from Δ*och1 mnn1* mutant cells as an enzyme source and the pyradylaminated core oligosaccharide (Man8GlcNAc-2PA) as an acceptor. The analysis of the reaction mixture by HPLC showed two new peaks later than the Man8GlcNAc2PA peak. Their retention times seemed to be identical to those identified as mannosylphosphorylated Man8GlcNAc2PA. We are now trying to purify these mannosylphosphate transferases.

We also analysed the O-linked oligosaccharide structure of the *S. cerevisiae mnn1* mutant and found the presence of mannosylphosphorylated Man α1,2 Man α1,2 Man. This indicated that *S. cerevisiae* has a mannosylphosphate transferase that transfers mannosylphosphate residue to Man α1,2 Man α1,2 Man. We are also trying to analyse this mannosylphosphate transferase activity.

1. Nishikido *et al.*

S12

Structures of the Phosphorylated N-Linked Oligosaccharides from the Δ*och1mnn1* Mutant of *Saccharomyces cerevisiae*

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The Δ*och1mnn1* mutant predominantly accumulates a single endoplasmic reticulum-form core oligosaccharide (Man8Glc-NAc2) [1]. It is possible that this mutant also accumulates a small amount of phosphorylated oligosaccharides which are limited in yeast.

In this study, we investigated the presence of phosphorylated oligosaccharides and their structures. N-linked oligosaccharides released from cell surface mannoprotein obtained from this mutant, were purified and their structures analysed after pyridylamination. The structures of three acidic oligosaccharides that mannosyl phosphate attached to the Man-8GlcNAc2 were determined using matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS), exo-a-1,2-mannosidase digestion and ^{1}H-NMR.

This work was performed as a part of the Research and Development Projects of Industrial Science and Technology Frontier Program supported by NEDO (New Energy and Industrial Technology Development Organization).

1. Y. Nakanishi-Shindo *et al.* (1993) *J Biol Chem* **268**: 26338–45.

S12

Purification to Homogeneity and Photoaffinity Labelling of Trehalose-6-P Synthase from Mycobacterium Smegmatis

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We previously reported that the partially purified trehalose-6-P synthase from *Mycobacterium smegmatis* could catalyse the transfer of glucose from either UDP-glucose or GDP-glucose to glucose-6-P to form trehalose-6-P and either UDP or GDP. Those studies showed that when UDP-glucose was the glucosyl donor, the synthase activity was greatly stimulated by the addition of polyanions such as RNA or heparin to the incubation mixture. A heparin chain with at least 18 monosaccharides was necessary for activation. However, when GDP-glucose was used as the glucosyl donor, polyanions had only slight stimulatory effects on the activity. Recently, we were able to purify this enzyme to apparent homogeneity using a variety of standard purification methods. The purified enzyme is still able to use various nucleoside diphosphate glucoses (i.e. ADPG, CDPG, GDPG, UDPG, TDPG) as glycosyl donors, depending on the presence of polyanion activator. On SDS-PAGE, the purified enzyme shows two protein bands of 61 and 59 kDa with the 61 kDa band being the major component. Exposure of the native protein to azido-UDP[^{32}P]-glucose resulted in the labelling of both proteins, and this labelling was inhibited by either cold UDPG or GDPG. The data indicate that the active site recognizes both UDPG and GDPC.

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S12

Burkholderia (Pseudomonas) Solonacearum Lipopolysaccharides: Structure-function Investigations

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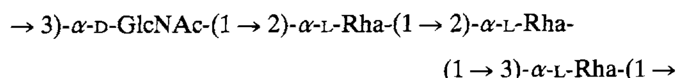
The scientific and practical interest in structure-function investigations of lipopolysaccharides (LPS) – the component of gram-negative bacteria outer membrane – arises from their action on the immune system of the macroorganism. The ability of LPS to stimulate the production of cytokines which are essential in bacterial and viral infections is known. LPS were isolated from cells of *Burkholderia (Pseudomonas) Solonacearum* by the phenol water procedure. The individual components-O-specific polysaccharide (O-PS), core-oligosaccharides (CO) and lipid A were obtained by mild acid degradation of LPS. The data of C NMR spectra showed that O-PS had a tetrasaccharide repeating unit:



$\rightarrow$ 3)-α-D-GlcNAc-(1 $\rightarrow$ 2)-α-L-Rha-(1 $\rightarrow$ 2)-α-L-Rha-(1 $\rightarrow$ 3)-α-L-Rha-(1 $\rightarrow$

LPS as well as O-PS and lipid A exert a pronounced effect on tumour necrosis factor, interleukin-1 and γ-interferon production. We showed that the injection of LPS, O-PS and CO into mice greatly reduced the volume and quantity of metastasis (70, 50 and 63% correspondingly).

S12

Promotion of Phagocytosis and Prevention of Phagosome-lysosome (P-L) Fusion in Human Peripheral Blood Monocytes by Serotype Specific Glycopeptidolipid (GPL) Antigen of *Mycobacterium avium* Complex (MAC)

Y. Takegaki, J. Fukui, N. Fujiwara, H. Fujii, J. Matsuda, H. Minami, S. Oka and *I. Yano*
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The *Mycobacterium avium* complex (MAC) is known to be one of the most important opportunistic pathogens associated with HIV infection (AIDS) and a typical intracellular parasite as well as *M. tuberculosis*. It is also characterized that *M. avium* infection induces immunosuppression especially in cellular immunity of host animals, and specific serotype-subspecies such as sero-4, -8 or -16 can frequently be isolated in human infection in AIDS. Furthermore, the prognosis after infection differs by the serotype, sero-4 shows heavy infection in general, while sero-16 shows rapid improvement. We have therefore, been interested in the immunomodifying activity of surface glycopeptidolipid (GPL) antigen. However, to date, no information has been available on the virulent factor related to intracellular bacterial killing. Recently we have tried to test the effect of various GPLs purified from the MAC complex on phagocytic processes of human peripheral blood monocytes (PBMC). We have used GPL-coated heat-killed staphylococcal cells phagocytosed by PBMC, and P-L fusion was estimated by the acridine orange staining of fused vesicles and bacteria. As a result, serotype-4 GPL showed strong phagocytosis promotion and marked inhibition of P-L fusion, while serotype-16 GPL showed neither promotion of phagocytosis, nor inhibition of P-L fusion in phagocytic cells. Serotype-8 GPL showed stimulation of both phagocytosis and P-L fusion, concomitantly. These effects may be due to an unknown interaction between specific carbohydrate chain and organella membranes, and serotype-4 GPL may be one of the possible virulent factors in MAC infection in human. Comparison with known possible virulent factors such as cord factor, trehalose monomycolate or sulfolipids will be reported.

S12

Expression of Mammalian Glycosyltransferases in the Yeast *S. cerevisiae*

S. Yoshida, S. Yamano, M. Suzuki, M. N. Fukuda, Y. Jigami and H. Sakai, M. Takeuchi and H. Ikenaga
Central Laboratory for Key Technology, Kirin Brewery Co., Ltd, Kanagawa 236, Japan.

Mammalian glycosyltransferases (such as GalT and GnT) and yeast mammosyltransferases (OCH1 and MNT1) are type II membrane proteins, whose N-terminal cytoplasmic and membrane spanning regions contain a Golgi retention signal (GRS). It has been reported that the mammalian galactosyltransferase (GalT) is retained in the ER, but not in the Golgi membranes, when expressed in yeast. Therefore, we constructed and expressed the yeast/mammal chimeric gene (*OCH1-GalT*) and studied whether the gene product localized in the Golgi membranes. The OCH1-GalT protein resides mainly in the low speed pellet (LSP), which contains vacuole, ER and plasma membranes. The protein also localizes in the periplasm, suggesting that the chimeric protein passes through the Golgi membranes. Moreover, we expressed another glycosyltransferase (*N*-acetylglucosaminyltransferase I, GnT-I) in the yeast and characterized the gene product. The GnT-I protein also localizes in LSP. We are currently examining the localization of GnT-I by using the ΔN-GnT-I which does not have GRS.

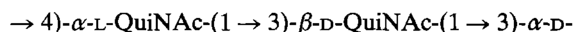
This work was performed as a part of the Research and Development Projects of Industrial Science and Technology Frontier Program supported by NEDO (New Energy and Industrial Technology Development Organization).

S12

Studies of *Pseudomonas fluorescens* Lipopolysaccharide

G. M. Zdorovenko, S. Veremeichenko and I. Ya. Zakharova
Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, Kiev, Ukraine.

The decisive role of lipopolysaccharides (LPS) in gram-negative bacteriaemia is generally known. This fact promotes investigations into these glycoconjugates. LPS was extracted from *Pseudomonas fluorescens* IMV 1433 (biovar I) cells. On mild acid hydrolysis of LPS lipid A, core oligosaccharide and O-specific polysaccharide (O-PS) fractions were obtained. Using GLC-MS and aminoacid analyser $C_{10:0}$, 3-OH$C_{10:0}$, $C_{12:0}$, 2-OH$C_{12:0}$, 3-OH$C_{12:0}$, $C_{16:0}$ fatty acids and GlcN, EtN-P and some aminoacids were identified in the lipid A fraction. The core contained Rha, Gal, Glc, KDO, GlcN, Ala and P. On the basis of NMR-spectroscopy, GLC-MS, etc, the structure of the O-PS chain was established as:


→ 4)-α-L-QuiNAc-(1 → 3)-β-D-QuiNAc-(1 → 3)-α-D-

Fuc4NAc-(1 →

LPS and O-PS preparations were active in the tests with homologous O-antiserum against microbial cells. Antioxidizing (56.9%) and antiradical (79.2%) biological activities of LPS were detected. The comparative studies on LPS, O-PS and core fractions showed the relationship of both activities with the O-PS part of the LPS macromolecule.

TUESDAY 22 AUGUST, MORNING

PLENARY LECTURES

S13. 8.00am

Glycopeptides as Oligosaccharide Mimics in Carbohydrate-Protein Interactions

K. Bock and M. Meldal
Department of Chemistry, Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Valby Copenhagen, Denmark.

The isolation, structural characterization and eventual subsequent synthesis of complex oligosaccharides, which are in general responsible for important biological interactions are individually a major research project which requires special skills and expertize, particularly when sialic acid or other labile residues are present in the parent structure.

We have successfully demonstrated that the much simpler glycopeptides, which can be prepared by easy and fully automated solid-phase (glyco)peptide synthesis, can mimic the above mentioned complex oligosaccharides in biological *in vitro* assays. These results are most likely due to the similarity in the 3-D structure of the two types of compounds.

Our progress in evaluating such an approach in the preparation of such signal molecules i.e. potential inhibitors using glycopeptides for the study of the interaction between the mannose 6-phosphate receptor and phosphorylated high-mannose N-linked oligosaccharides or for selectin binding simulating sialyl Le^{x} type oligosaccharides will be presented. Furthermore the future approach using (glyco)peptide libraries to identify the active compounds will be discussed and evaluated.

The lecture will focus both on synthetic and conformational aspects of the problem.

S14. 8.30am

Enzyme Replacement and Gene Therapy for Gaucher Disease

Roscoe O. Brady
National Institutes of Health, Bethesda, Maryland 20892, USA.

Gaucher disease is the most prevalent inherited metabolic storage disorder of humans. The condition is caused by insufficient activity of the enzyme glucocerebrosidase. Studies carried out in 1973 revealed that the intravenous injection of purified human placental glucocerebrosidase caused a reduction of the accumulated glucocerebroside in the liver and in the blood of patients with the disorder. In order to carry out more extensive clinical trials, two major obstacles had to be overcome. First, it was necessary to develop a large-scale purification procedure to obtain sufficient glucocerebrosidase for these studies. Second, the enzyme had to be modified so that it was targeted to tissue macrophages where glucocerebroside is stored. The latter goal was accomplished by sequentially treating glucocerebrosidase with exoglycosidases that cleaved N-acetylneuraminic acid, galactose, and N-acetylglucosamine residues producing mannose-terminal enzyme. This glycoform of glucocerebrosidase is highly effective clinically. Patients with Gaucher disease treated with this preparation show dramatic improvement of anaemia and thrombocytopenia, reduction of the size of the enlarged spleen and liver, and correction of skeletal deterioration. We have recently completed preclinical studies on the transfer of the normal human glucocerebrosidase gene to stem cells of patients with Gaucher disease. These investigations have led to approval by the NIH Recombinant Advisory Committee for a Phase 1 trial of gene therapy in patients with Gaucher's disease. It is expected that this study will be initiated soon.

Supported by Genzyme Corporation.

S15. 9.00am

The Functional Role of the Polypeptide Chain and the Oligosaccharide Chains of the Sea Urchin Egg Receptor for Sperm

S. T. Dhume, K. Ohlendieck, R. Stears and *William J. Lennarz*
Department of Biochemistry and Cell Biology, State University of New York at Stony Brook, Stony Brook, NY 11794-5215, USA.

The sea urchin egg receptor for sperm has been cloned and sequenced. The intact receptor was purified to homogeneity from *S. purpuratus* eggs. Because of prior evidence implicating the oligosaccharide chains of the receptor in sperm/egg adhesion we examined the carbohydrates and found that the receptor is a sulfated and highly glycosylated protein. The N-

and O-linked oligosaccharide chains of the receptor were cleaved from the protein and separated from each other. The O-linked oligosaccharides were further fractionated by anion exchange chromatography. Using a competition bioassay that measured the ability of these oligosaccharide chains to inhibit fertilization, it was found that the N-linked chains were devoid of inhibitory activity. Rather, the inhibitory activity was localized to the O-linked chains, with the most highly charged, sulfated chains showing the highest inhibitory activity. The bioactive oligosaccharides were assayed for binding to sperm. The results of the binding assay, coupled with the fertilization bioassay, indicate that the oligosaccharides inhibit fertilization by binding to acrosome-reacted sperm. The bioactive oligosaccharide lacked species specificity in fertilization bioassays, unlike the intact receptor and a recombinant aglycoprotein containing only the extracellular domain of the receptor. Since previous work showed that the recombinant protein inhibits fertilization species specifically and binds to acrosome-reacted sperm, a two-step model of sperm-egg interaction is proposed.

Supported by NIH Grant HD18590 and Takara Shuzo Co.

S13. GLYCOMIMETICS

Chairs: Ole Hindsgaul, Tomoya Ogawa

S13. 9.50am

Novel Photoreactive Carbohydrate Probes for Photoaffinity Biotinylation

Y. Hatanaka
Research Institute for Wakan-Yaku, Toyama Medical and Pharmaceutical University, Sugitani 2630, Toyama, 930-01 Japan.

Photoaffinity labelling is an important chemical method in the field of modern molecular biology and much attention has been devoted to the application of this method to the identification of ligand binding regions. Due to the low yield of cross-linking, however, photoaffinity labelling experiments usually encounter the thorny problem of how to isolate labelled components from tremendously complex mixtures. We have developed novel photoreactive carbohydrate derivatives carrying a photolabile aryl diazirine and a biotin moiety in order to make use of avidin-biotin technology for the selective manipulation of photolabelled components.

A successful application of this reagent for the identification of substrate binding-sites within β-1,4-galactosyltransferase (GT) protein will be exemplified. A carbene generating a biotinylated *N*-acetylglucosamine derivative was specifically incorporated into bovine GT protein. Based on the enzyme-catalysed signal amplification of the avidin-biotin complex, a highly sensitive visualization of the photoaffinity biotinylated GT was performed by the chemiluminescent detection method. Combined use of this reagent with an immobilized avidin was also successful for the selective retrieval of photolabelled GalT from a reaction mixture. Synthesis and application of other carbohydrate probes of this category will also be discussed.

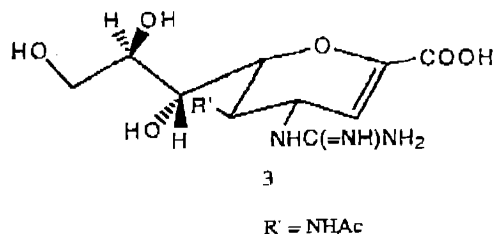
S13. 10.15am

Carbohydrate Analogues as Biological Probes

Mark von Itzstein
Department of Medicinal Chemistry, Victorian College of Pharmacy, Monash University, 381 Royal Parade, Parkville 3052, Victoria, Australia.

Carbohydrate-metabolizing enzymes may provide useful targets for the discovery of clinically-useful novel carbohydrate-based therapeutics. We have recently reported the design, synthesis and biochemical evaluation of a number of potent inhibitors of the glycohydrolase, sialidase [1–4]. Indeed, some of these inhibitors have shown significant *in vivo* activity in a number of animal models [1, 5].

Our approach towards the design, synthesis and biochemical evaluation of such compounds, as well as some of our more recent studies on a potential anti-influenza drug candidate, 4-guanidino-Neu5Ac2en (**3**) [6] will be discussed.



R' = NHAc

1. von Itzstein M, *et al.* (1993) *Nature* **363**: 418–23.
2. Holzer CT, *et al.* (1993) *Glyconjugate J* **10**: 40–44.
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5. Ryan DM, Ticehurst J, Dempsey M, Penn CP (1994) *Antimicrob Ag Chemotherap* **38**: 2270–5.
6. Pegg MS, von Itzstein M (1994) *Biochem Mol Biol Int* **32**: (5) 851–8.

S13. 10.40am

Glycodendrimers: New Powerful Inhibitors of Carbohydrate-protein Interactions

W. K. C. Park, B. Kratzer, D. Zanini, Q. Wu, S. J. Meunier and *R. Roy*
Department of Chemistry, University of Ottawa, Ottawa, ON, Canada K1N 6N5.

Low protein receptor affinities towards carbohydrate ligands have triggered the design of multivalent carbohydrate clusters having improved binding properties. Such artificial glycoforms are finding a wide range of applications as high avidity inhibitors in cell-cell interactions and in viral and bacterial infections by blocking haemagglutinins and adhesins. Refined inhibitors design has recently included glycodendrimers which are multi-branched semi-macromolecules. Similarly to their glycopolymer counterparts, dendritic sialosides with valencies between 2 and 16 have shown thousand fold increases in inhibition of influenza virus haemagglutinins.

Dendritic glycopeptides of model glycosides, including sialosides, were initially prepared by solid-phase chemistry using L-lysine branching units and *N*-chloroacetylglycylglycine spacers. The valency growth in these glycodendrimers were consequently 2^n, n representing the dendrimer generation. Subsequently, gallic acid and ethylene glycol spacers with 3^n valencies were chosen as core branching units. With gallic acid backbones, hyper-branched dendritic sialosides and lactosides with valencies of three and nine residues were synthesized using amide couplings. The synthesis of GM3 trisaccharide derivatives in forms suitable for dendrimer synthesis will also be described.

Another approach, based on phosphoramidite chemistry, has been used to prepare hyper-branched dendritic phosphotriester glycosides. This novel strategy has allowed the construction of a large number of dendritic clusters by the tethering of subfragments. All the resulting glycodendrimers form precipitin bands with plant lectins in agar double diffusion experiments.

S13. 11.05am

Design and Preparation of Novel Polymer Supports for Enzymatic Glycoconjugate Syntheses

Shin-Ichiro Nishimura, Kuriko Yamada and Miwa Kanemaru
Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo, 060 Japan.

Water soluble polymers as high performance primers for the enzymatic syntheses of glycoconjugates have recently received ever increasing attention because of their high efficiency in glycosylation reactions and simple procedures for the purification of products [1].

In the present sudy, we report a novel and efficient method for the preparation of unique *primer polymers* bearing α-chymotrypsin sensitive structures in their spacer-arm moieties. Firstly, GlcNAc monomers having a phenylalanine residue in each aglycon were synthesized by glycosidation of oxazoline derivatives with protected amino acid derivatives. Deprotection by the Zemplen procedure and copolymerization of these sugar monomers proceeded smoothly to afford the *primer polymers* in high yields. Galactosylation of these polymers was carried out with bovine milk galactosyl transferase and gave LacNAc-polymers in quantitative yields. A useful LacNAc derivative having a terminal amino group was released from the polymer chains by treating with α-chymotrypsin in Tris-HCl buffer (pH 7.8) at 40 °C and purified by simple chromatography on a Sephadex G-10 column. The versatility of this reactive oligosaccharide derivative as a tool for the preparation of *neoglycoconjugates* is under investigation.

1. Nishimura S-I, Matsuoka K, Lee YC (1994) *Tetrahedron Lett* **35**: 5657.

S13. 11.25am

Synthesis of Complex Carbohydrates by a Strategy Employing both Chemical and Enzymatic Methods

R. Walton, H. Fujimoto, M. Isomura and K. Ajisaka
Meiki Institute of Health Science, Meiji Milk Products, Co., Ltd, 540 Naruda, Odawara 250, Japan.

The focus of the research now being conducted in our laboratory is the synthesis of complex carbohydrates of the general structure Galβ1-4GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc employing both enzymatic and chemical methods.

The core structure of a wide variety of complex carbohydrates consists of a β-linked mannose-chitobiose trisaccharide. A considerable amount of the literature has been dedicated to construction of this basic structure, largely due to the difficulty in obtaining the β-linkage between mannose and chitobiose.

The first portion of this presentation deals with the chemical synthesis of this core structure as a fully and differentially protected unit ready for extension at either the reducing or non-reducing end. The key reaction in this sequence involves a double inversion which converts the β-linked galactosyl portion to the analogous β-linked mannosyl structure [1, 2].

The second portion of this presentation deals with the use of this acceptor to synthesize complex carbohydrates. GlcNAcβ1-2Man was enzymatically synthesized using *N*-acetyl-β-glucosaminidase from Jack Bean to obtain the disaccharide donor, and galactose was transglycosylated using β-galactosidase from *Bacillus circulans* to obtain the trisaccharide donor.

This work was performed as a part of the Research and Development Project of the Industrial and Technology Program supported by the New Energy and Industrial Technology Development Organization.

1. Alais J, David S (1990) *Carbohydr Res* **201**: 69.
2. Sato K *et al*. (1992) *Chemistry Lett* 1469.

S13. 11.40am

A Synthetic Approach to Glycopeptides from the Carbohydrate-Protein Linkage Region of Proteoglycans

Klaus W. Neumann[1], Jun-ichi Tamura[1] and Tomoya Ogawa[1,2]
[1]*The Institute of Physical and Chemical Research (RIKEN), Wako-shi, Saitama, 351-01 Japan.*
[2]*Graduate School for Agriculture and Life Sciences, University of Tokyo, Yayoi, Bunkyo-ku, Tokyo, 113 Japan.*

The attachment of specific glycosaminoglycan chains (e.g. chondroitin, chondroitin sulfate, heparin) during the biosynthesis of proteoglycans is of crucial importance to the displayed biological activities. Thus, their availability and further characterization is a major task on the path to understanding which structural features give rise to their specific activities and to reveal factors governing their biosynthesis. This prompted us to investigate synthetic approaches towards the synthesis of glycopeptides from the carbohydrate-protein linkage region of proteoglycans. Described herein is our approach to combine the common tetrasaccharide unit, GlcAGalGalXyl, of O-linked proteoglycans with peptide fragments of the core protein. First, the GlcAGalGalXyl tetrasaccharide was synthesized in a stereocontrolled manner employing different protocols for the activation of thiomethyl donors (NIS/TfOH, $CuBr_2/Bu_4NBr$/AgOTf). On the other hand, peptides (1-4 amino acid residues) were synthesized by chemical means, using Fmoc- or Boc-strategy, to give the required acceptor compounds for the glycosylation between the tetrasaccharide and the serine residue of the peptide unit. Our investigations showed the tetrasaccharide imidate to be a well suited donor for such reactions, and one which could be activated under different conditions (TMSOTf, $BF_3 \cdot Et_2O$) to give completely protected glycopeptide structures in good yields. In the final steps of the synthesis deprotection was performed using mild conditions to avoid any kind of undesired side-reaction.

In summary, new and unique glycopeptide structures were synthesized in an efficient manner. The obtained material should serve either as transferase acceptors or standards for transferase products during biosynthetic studies on glycosaminoglycan synthesis of proteoglycans.

S13. 11.55am

Random Glycosylation of Unprotected Carbohydrates: A Strategy for the Synthesis of Oligosaccharide Libraries

Osamu Kanie[1], Frank Barresi[1], Yili Ding[1], Jill Labbe[1], Albin Otter[1], L. Scott Forsberg[1], Beat Ernst[3] and *Ole Hindsgaul*[1]
[1]*Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2.*
[2]*Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia 30602-7229.*
[3]*Carbohydrate Group, Ciba-Geigy Central Research, 4002-Basel, Switzerland.*

An unexpected and remarkable lack of regiospecificity was observed on chemical glycosylation of completely unprotected dissaccharides using per-*O*-benzylated trichloroacetimidates as glycosyl donors in DMF: all possible α-linked trisaccharides were formed even though the acceptors contained four primary and two secondary OH-groups. The results suggest that random glycosylation of unprotected sugar acceptors may be a strategy for the preparation of oligosaccharide libraries.

S13 POSTERS

S13

A New Synthetic Inhibitor of Glycosyl Transferase

K. Hatanaka and H. Takeshige
Department of Biomolecular Engineering, Tokyo Institute of Technology, Faculty of Bioscience and Biotechnology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226, Japan.

Many inhibitors of the processing enzymes (glycosidases) for N-linked oligosaccharide are known. For example, swainsonine inhibits mannosidase II, castanospermine inhibits glucosidase I and II, and so on. On the other hand, there are not many glycosyl transferase inhibitors such as tunicamycin which inhibit the first step in the lipid-linked saccharide pathway.

In this investigation we attempted to prepare a new inhibitor of glycosyl transfer reaction by chemically attaching p-styrenesulfonyl group to the 5′-OH of uridine followed by polymerization. Thus, 2′,3′-*O*-isopropylideneuridine 5′-p-styrenesulfonate was synthesized and polymerized. However, the deprotection of the polymer gave an irregular structure due to a side reaction. On the other hand, acetyl groups of poly(2′,3′-di-*O*-acetyluridine 5′-p-styrenesulfonate) were removed under mild condition without side reactions. This uridine-containing polymer was tested against the galactosyl transferase that synthesizes a lactose in the presence of α-lactalbumin. The acceptor used in these reactions was p-nitophenyl β-D-glucopyranoside. The extent of the glycosyl transfer reaction was evaluated by HPLC measurement with a UV detector. The polymeric compound inhibited this enzyme by 75% requiring 120 μM which is only 1% of the concentration of glycosyl donor substrate (UDP-Galactose).

S13

Synthesis of 2-acetamido-2-deoxy-(1 → 6)-β-D-glucopyranan Derivatives

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[1]*Department of Biomolecular Engineering, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226, Japan.*
[2]*Graduate School of Science, Hokkaido University, Sapporo 060, Hokkaido, Japan.*
[3]*Faculty of Engineering, Saitama University, 255 Shimo-ohkubo, Urawa-shi 338, Japan.*

Ring-opening polymerization of 1,6-anhydro-2-deoxy-3,4-di-*O*-benzyl-2-phthalimido-β-D-glucopyranose (1) yielded stereoregular oligomers having, as an average, five to seven (compound 2) degrees of polymerization. ^{1}H NMR spectrum of 2 showed an anomeric proton signal at 5.21 ppm with a large coupling constant ($J_{1,2}$ = 7.92 Hz). The large $J_{1,2}$ value means that the relationship between H-1 and H-2 proton is *anti*. Thus, it was concluded that 2 was stereoregular 2-deoxy-3,4-di-*O*-benzyl-2-phthalimido-(1 → 6)-β-D-glucopyranan. Deprotection of 2 and consequent acetylation gave novel 2-acetamido-2-deoxy-(1 → 6)-β-D-glucopyranan (3).

In addition, it was attempted to copolymerize 1 with 1,6-anhydro-2,3,4-tri-*O*-benzyl-β-D-glucopyranose (4), and the monomer reactivity ratios were $r_1 = 0.0214$ and $r_4 = 1.06$. These values indicated that the reactivity of 1 is as high as 4 against the highly-reactive cation derived from 4, while it is low against the cation derived from 1. It can be speculated that the reaction of 1 with the 1 derived cation was restricted by steric hindrance between the whole propagating oligosaccharide chain end of 1 and phthalimido groups of 1. Copolymerizations of 1 with other 1,6-anhydro-sugars are also possible to give artificial glycans which mimic natural glycans.

S13

Sucrose Phosphorylase is a Glucosyltransferase Suitable for Synthesis of Various Phenol Glucosides

Satoshi Kitao[1], Tatsuo Horiuchi[1] and Hiroshi Sekine[2]
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[2]*Noda Institute for Scientific Research, 399 Noda, Noda-shi, Chiba 278, Japan.*

Sucrose phosphorylase (SPase, [EC 2.4.1.7]) catalyses a reversible transglucosylation of sucrose. SPase from *Leuconostoc mesenteroides* was found to have a broad acceptor specificity and the glucosyl moiety of sucrose could be transferred to phenolic or alcoholic OH groups of various phenol-related compounds such as hydroxybenzenes, hydroxybenzoic acids and hydroxybenzyl alcohols. Phenolic OH groups adjacent to hydroxy, hydroxymethyl, or carboxyl groups were in a suitable position for accepting glucosyl moiety. When hydroquinone (1,4-dihydroxybenzene) was used as an acceptor, the transfer ratio was more than 80% (molar basis) and the product was identified as hydroquinone-*O*-α-D-glucopyranoside (α-arbutin). Catechins, which possess a lot of phenolic OH groups, were also good glucosyl acceptors and their transfer ratios of the reaction were more than 40% (molar basis). In the case of (+) catechin, the main transfer product was (+)-catechin 3′-*O*-α-D-glucopyranoside (CG). In the reaction with (−)-epigallocate-

chin galate (EGCg) as an acceptor, two staple products were observed and their structures were identified as (−)-epigallocatechin gallate 4′-*O*-*α*-D-glucopyranoside (EGCgG-1) and (−)-epigallocatechin gallate 4′,4″-*O*-*α*-D-diglucopyranoside (EGCgG-2). These results show that SPase tends to transfer the glucosyl moiety of sucrose to the pyrogallol ring of catechins.

The resistances of *α*-arbutin and CG to browning by light irradiation were extremely increased compared to those of their aglycones. The stability of EGCg in water was markedly improved by the addition of one glucosyl moiety (EGCgG-1, EGCgG-2). The solubilities of catechin glucosides in water were over 25 times higher than those of their aglycones. SPase is a useful enzyme for the improvement of the physical properties of phenolic compounds by glucosylation.

S13

Artificial Glycopolypeptides: Simple Synthesis via *β*-*N*-Glycosidation of *N*,*N*′-Diacetylchitobiose on to Poly(glutamic Acid)

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[1]*School of Agricultural Sciences, Nagoya University, Chikusa, Nagoya 464-01, Japan.*
[2]*Tokyo Institute of Technology, Tokyo, Japan.*
[3]*Shizuoka University, Shizuoka University, Japan.*

Poly(glutamic acid) substituted with *N*,*N*′-diacetylchitobiose through *β*-N-glycoside bond, Poly(GlcNAc*β*1 → 4GlcNAc-*β*1 → N-Gln/Glu), was synthesized as a model substance of glycopolypeptides. The reducing terminal of *N*,*N*′-diacetylchitobiose was reduced with ammonium hydrogen carbonate to give its glycosylamine. Pendant carboxyl groups of poly(L-glutamic acid) were activated with peptide coupling reagents such as BOP and HOBt in dimethyl sulfoxide, and then condensed with the glycosylamine in dimethyl sulfoxide at room temperature. The condensation product was purified by gel-filtration chromatography using Sephadex. The oligosaccharide was incorporated into 27 mol % of carboxylic acids in the poly(glutamic acid) as estimated by ^{1}H-NMR spectroscopy.

The glycopolypeptide inhibited haemagglutination activity of wheat germ agglutinin (WGA) much more strongly (about 10^5 times) than *N*,*N*′-diacetyl-chitobiose itself. The high activity is due to the cluster or high density effect of the glycopolypeptide.

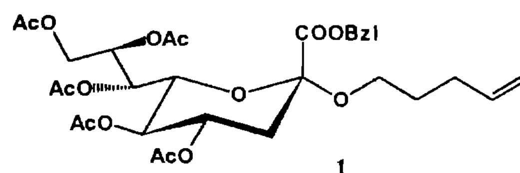
S13

Design and Synthesis of Cell Adhesive Glycopeptide Ligands

Masao Matsuda[1], Miwa Kanemaru[1], Shin-Ichiro Nishimura[1] and Norio Nishi[2]
[1]*Division of Biological Science, Graduate School of Science, and* [2]*Division of Ecological Science, Graduate School of Environmental Earth Science, Hokkaido University, Sapporo, 060 Japan.*

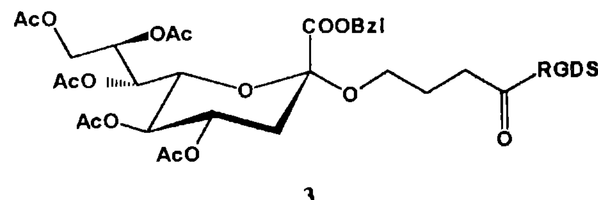
Sialic acid and its derivatives are known as effective inhibitors of infection by the influenza virus in animal cells. Our interest is now aimed at combining the different biological activities of sialic acid and RGDS which is known as the ligand for macrophages or neutrophiles. In the present study, we designed and systematically synthesized a novel class of a hybrid glycopeptide.

A key sialic acid derivative 1, pentenyl benzyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-a-D-galacto- 2-nonulopyranosylonate, was prepared from sialic acid in moderate yield. A RGDS derivative 2 having a reactive-terminal amino group was prepared by the usual stepwise synthesis. A terminal C-C double bond in the pentenyl group of the sialic acid derivative was selectively oxidized with potassium permanganate to give a carboxyl component. This sialic acid derivative was coupled with the above tetrapeptide to afford the targeted glycopeptide-ligand 3.



Arg(N•NO$_2$)GlyAspSer(Bzl)OBzl

2



S13

Induction of Cell Differentiation and Apoptosis into Human Malignant Tumour Cell Lines by Drugs Affecting Ganglioside Biosynthesis

H. Nojiri[1], H. Hori[2] and S. Nojima[1]
[1]*Faculty of Pharmaceutical Science, Teikyo University, Japan.*
[2]*Department of Biological Science and Technology, Faculty of Engineering University of Tokushima, Japan.*

Sodium butyrate can induce cell differentiation in some human epithelial carcinoma cell lines. Characteristic changes of gangliosides were observed during the differentiation of human oesophagial carcinoma cell line ES-2 and human colonic carcinoma cell line HCT 116. Ganglio-series ganglioside G_{M3} increased remarkably with concomitant decrease of neolacto-series gangliosides.

When ES-2 and HCT 116 cells were treated with Brefeldin A (BFA), which has been reported to inhibit *de novo* biosynthesis of neolacto-series glycosphingo-lipids with a concomitant increase of G_{M3}, morphological changes showing cell differentiation were observed. DNA-ladder, which is one of the markers for apoptosis, was also observed in BFA-treated cells following such morphological changes. Ganglioside analysis of BFA-treated cells showed a remarkable increase in G_{M3} and decrease in neolacto-series gangliosides with longer carbohydrate moieties. Studies with other drugs affecting ganglioside biosynthesis suggested that suppression of neolacto-series gangliosides and an increase in G_{M3} might be essential for differentiation of human epithelial carcinoma cell lines and that such changes in gangliosides might be promoting differentiation of the cells.

S13

An Asymmetric Synthesis of L-Azafucose and Azafucosylceramide from L-Alanin

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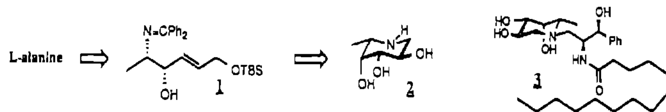
Azasugars are potent inhibitors of glycoconjugate processing enzymes (glycosidases and glycosyltransferases). We have applied our sequential reduction-alkenylation methodology, followed by OsO_4-catalysed cis-hydroxylation to convert L-alanine to azafucose. Compound **1** was obtained with excellent stereoselectivity (>20:1, 70% yield) from L-alanine and TBS-oxypropenyl lithium. Stereoselective oxygenation of the double bond, orthogonal manipulation of the protecting groups and cyclization provided azafucose **2**. We also propose a novel inhibitor azafucosylceramide **3**, where a glycosyl donor mimic (azafucose 2) is covalently linked to an acceptor mimic (threo-ceramide). This approach should provide a specific inhibitor of the fucosylceramide synthase, an enzyme expressed exclusively in adenocarcinoma of the lung, stomach and colon. The compound **3** represents the first generation of the *sequence specific inhibitors* of glycosyltransferases.

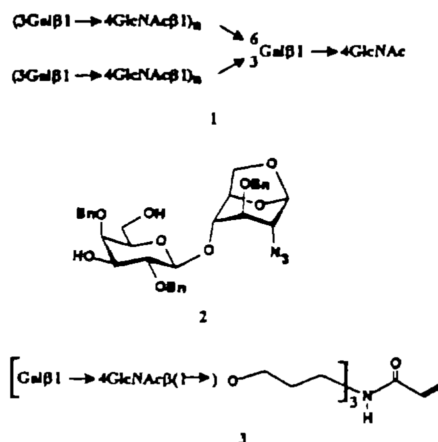


S13

Clustered Lactosaminoglycan-Mimetics

Tetsuro Tsuda[1], Tetsuya Furuike[2], Kimito Washiya[1] and Shin-Ichiro Nishimura[1]
[1]*Division of Biological Sciences, Graduate School of Science and* [2]*Division of Ecological Science, Graduate School of Environmental Earth Science, Hokkaido University, Sapporo, 060 Japan.*

To investigate the cluster effect of branched lactosaminoglycan 1, two lactosaminoglycan-mimetics were designed and synthesized. A key intermediate 2 was modified by the chemical introduction of GlcNAc residues and followed by enzymatic elongation of Gal residues to give a hexasaccharide derivative. Compound 3 having triple chains of LacNAc residues was systematically prepared from a known LacNAc donor and the convenient cascade building block, 'bis-homotris' derivative, and converted into a new type of clustered LacNAc polymer by radical copolymerization.



S13

Highly Regio- and Stereo-selective Synthesis of Glycosaminoglycan Oligosaccharides

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Division of Medicinal and Natural Products Chemistry, College of Pharmacy, Universtiy of Iowa, Iowa City, IA 52242, USA.

Glycosaminoglycans are involved in a number of biological events by interacting with a diverse group of proteins. For example, heparin, heparan sulfate and dermatan sulfate inhibit the serine protease thrombin by binding to antithrombin III and heparin cofactor II, causing an inhibition in blood coagulation. Synthetic oligosaccharide sequences of these polydisperse glycosaminoglycans, corresponding to protein binding sites, are of interest to our group. These sequences are ideally suited for interaction with a number of important proteins. The α-L-idopyranuronic acid (α-L-IdoAp) is a key constituent of glycosaminoglycans and their synthetic oligosaccharide mimetics. L-IdoAp alternates with hexosamine residues to form linear chains that are *N*-sulfated. *O*-sulfated and *N*-acetylated. Our approach for synthesis of α-L-IdoAp donors and/or acceptors, is based on an orthogonally stable protecting group strategy. Thus, starting from D-glucurono-3,6-lactone, a versatile L-IdoAp-synthon is developed in a fully regio- and stereo-controlled manner. Stereoselective glycosidation reactions should then afford the target oligosaccharide structures. New approaches for the synthesis C-glycosides of acidic monosaccharides will also be discussed.

S14. MOLECULAR GENETIC BASIS OF SPHINGOLIPIDOSIS AND NEURONAL DISEASES

Chairs: John O'Brien, Yoshiyuki Suzuki

S14. 9.50am

Intracellular Hydrolysis of Lissamine-Rhodamine Glycosphingolipids to Ceramide which is Converted to Sphingomyelin that is Secreted into the Culture Medium: Application to Diagnosis, Prevention and Gene-Therapy of Lipid Storage Diseases

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Lissamine rhodamine (LR) or sulforhodamine (SR), polar fluorescent probes were linked via a 12-carbon fatty acid to glycosphingolipids and sphingomyelin (SPM). These were taken up by cultured cells and hydrolysed in the lysosomes to LR (or SR) ceramide which left the lysosomes and was converted, probably in the Golgi, to LR-sphingomyelin. The LR-SPM was trafficked to the plasma membrane and secreted into the culture medium. The quantity of LR-SPM in the medium of normal cells related to the intralysosomal catabolism of the LR-glycolipids. In contrast, in cells derived from patients with lipid storage diseases, in which defects in lysosomal hydrolases prevent the formation of LR-ceramide, LR-SPM is not formed or found in the culture medium. This difference between normal and lipidotic cells permitted diagnosing lipid storage diseases and characterizing disease subtypes (e.g. juvenile or adult) by quantifying the LR-SPM in the medium. Prevention of birth of lipidotic infants could be aimed at by prenatal diagnosis using intact amniocytes and by characterizing embryos at the preimplantation stage of *in vitro* fertilization. Towards gene therapy of lipid storage diseases a procedure was worked out for fluorescence-based selection of lipidotic cells which have been 'normalized' by infection with a retroviral vector containing the normal gene. The normalized cells were isolated in the fluorescence-activated cell sorter (FACS) and separated from those which have not incorporated the gene and consequently did not express enzymatic activity. In summary, the advantage of the use of LR (SR) sphingolipids is in their facilitating elimination of lysosomal hydrolysis products from the cells and by providing an evaluation of intracellular catabolism of glycosphingolipids by quantifying the LR-SPM in the culture medium while maintaining the cells intact.

S14. 10.20am

Targeted Disruption of the Mouse Acid β-Galactosidase Gene: An Animal Model for G_{M1}-gangliosidosis

J. Matsuda[1], O. Suzuki[1], A. Oshima[2], H. Sakuraba[2], Y. Suzuki[2], T. Asano[1] and M. Naiki[1]
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[2]*Department of Clinical Genetics, the Tokyo Metropolitan Institute of Medical Science, Tokyo 113, Japan.*

G_{M1}-gangliosidosis and Morquio B disease are lysosomal storage diseases caused by allelic mutations of the acid β-galactosidase gene. Here we generated a mouse model for G_{M1}-gangliosidosis by gene targeting in embryonic stem (ES) cells. To disrupt the β-galactosidase gene, we used a targeting vector containing a neo^r cassette inserted into exon 15 and a herpes simplex virus thymidine kinase gene at the downstream side of the construct. TT2 ES cells [1] and an isogenic TT2 genomic library [2] were used in this experiment. Out of 258 G418 and ganciclovir-resistant clones picked, 3 ES cell clones were determined as homologous recombinants and through generating chimeras the disrupted β-galactosidase gene was transmitted to their progeny. By mating of mice heterozygous for the disrupted β-galactosidase gene, homozygous mice were obtained. β-Galactosidase activities of mouse tails were assayed using 4-methylumbelliferyl-β-galactoside as a substrate. The enzyme activities were $3.7 \pm 0.6\,\mathrm{nmol\,mg^{-1}}$ protein per h (mean ± SD, $n = 5$) in homozygous mutants, 27.7 ± 5.9 ($n = 8$) in heterozygous mutants and 65.8 ± 16.1 ($n = 5$) in their wild-type litter mates. These data showed that the targeted gene disruption had resulted in a null allele and the enzyme activities were essentially absent in homozygous mutant mice. Although pathological examinations are still in progress, preliminary data showed a high vacuole accumulation inside the cytoplasm of peripheral leukocytes from homozygous mutants. To date, five homozygous mutant mice have been born and are apparently normal and healthy, at least until 2 months of age. It could be possible that in the case of mice the deficiency of β-galactosidase causes slowly progressive syndrome with later onset. The availability of mice lacking β-galactosidase should help to study the pathogenesis and therapeutics of G_{M1}-gangliosidosis.

1. Yagi T *et al*. (1993) *Analyt Biochem* **214**: 70–76.
2. Yagi T *et al*. (1993) *Analyt Biochem* **214**: 77–86.

S14. 10.50am

Prosaposin: a Multifunctional Protein with Important Roles in Glycolipid Metabolism

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[2]*Department of Experimental Medicine, 'La Sapienza', University of Rome, Rome, Italy.*

Prosaposin, the precursor of saposins A, B, C and D is a multifunctional protein. Historically, prosaposin was first identified as the precursor of the four saposin activator proteins which interact with lysosomal enzymes or glycolipid substrates to accelerate their hydrolysis. Absence of saposins leads to fatal lysosomal storage diseases in humans. In addition to its lysosomal precursor role prosaposin exists as an uncleaved protein in secretory fluids and as an integral membrane protein in several cell types. Prosaposin also appears to function as a carrier protein transporting gangliosides and cathepsin D from one cellular location to another. Finally, prosaposin has recently been identified as a potent neurotrophic factor with activity in the nanomolar range, binding to high affinity receptor and activating a tyrosine kinase cascade. The active region has been pinpointed to a 12 amino acid residue stretch in the saposin C domain. A review of salient properties of this important protein will be presented.

S14. 11.20am

Sphingolipid Ceramide *N*-Deacylase: An Enzyme that Cleaves the *N*-acyl Linkage of Ceramides in Various Glycosphingolipids as well as Sphingomyelin to Produce their Lyso Forms

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Lysoglycosphingolipids (LysoGSLs), which are GSLs *N*-deacylated at the ceramide moiety, have been detected in normal tissues at very low levels, but are accumulated in inherited sphingolipid storage diseases. Several lines of evidence have suggested the biological significance of lysoGSLs in cell activities [1]. However, the molecular mechanism of lysoGSL generation *in situ* remains unclear. Recently, Hirabayashi *et al.* reported the presence of lysoGSL-generating hydrolase activity in actinomycetes [2]. The enzyme was, however, difficult to solubilize from the cells, and thus the enzyme protein has not yet been characterized. We report a novel enzyme that hydrolyses the *N*-acyl linkage between fatty acids and sphingosine bases in ceramides of various sphingolipids. The enzyme was purified about 150-fold with 30% recovery from the culture filtrate of a newly isolated bacterium (*Pseudomonas* sp. TK4) by ammonium sulfate precipitation followed by several steps of HPLC. The purified enzyme preparation was completely free of exoglycosidases, sphingomyelinase and proteases, and showed one major band at a position corresponding to a molecular mass of 52 kDa on SDS-PAGE. The enzyme shows quite wide specificity, i.e. it hydrolyses both neutral and acidic GSLs including sulfatide, and simple GSLs cerebrosides to polysialogangliosides such as GQ1b. Furthermore the enzyme also hydrolyses sphingomyelin to produce the respective lyso form. This is the first report describing the generation of lysosphingomyelin from sphingomyelin by a specific hydrolase. However, the enzyme shows hardly any activity on ceramides, indicating that it is completely different from the ceramidase (EC 3.5.1.23) reported previously. This enzyme, which is tentatively named sphingolipid ceramide *N*-deacylase (SCDase), should greatly facilitate the further study of sphingolipids as well as lysosphingolipids.

1. Hannun YA, Bell RM (1987) *Science* **235**: 670–74.
2. Hirabayashi Y, Kimura M, Matsumoto M, Yamamoto K, Kadowaki S, Tochikura T (1988) *J Biochem* **103**: 1–4.

S14. 11.40am

B Glycolipid Antigens in Fabry Patients Blood Group B Secretors and Non-Secretors

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[1]*Center for Inherited Metabolic Diseases, 1st School of Medicine, Charles University, 128 53 Prague 2, Czech Republic.*
[2]*Institute of Haematology and Blood Transfusion 120 00 Prague 2, Czech Republic.*

Deficiency of lysosomal α-galactosidase (EC 3.2.1.22) in Fabry disease (McKusic 30150), leads to the progressive accumulation of glycosphingolipids with terminal α-galactose (mainly globotriaosylceramide Gb_3 Cer and galabiosylceramide Gb_2Cer) in lysosomes of most tissues and fluids of the body. Increase in B blood group glycolipids, however, has been proved only in the pancreas in one patient [1].

We have investigated glycosphingolipids in urinary sediments and tonsillar tissue of the Fabry patients and in healthy individuals whose blood group B secretors and non-secretors by direct anti-B blood group immunodetection on TLC plates.

A significant accumulation of B group glycolipid against controls was found in the tonsillar tissue of Fabry patients who were blood group B secretors. This points to the existence of its storage even beyond the glandular tissue. The B glycolipids were consistently found to be increased in the urinary sediment of four Fabry patients, who were blood group B secretors. Conversely, they were absent in the urinary sediment of one patient, a blood group B non-secretor. Concentration of Gb_3Cer/Ga_2Cer was unaffected by the secretory status. In healthy individuals – secretors, B glycolipids were present only in traces. We conclude that the secretory status is responsible for an increase in B group glycolipids in the urinary sediment in alpha-galactosidase deficiency.

1. Wherrett JR, Hakomori S-I (1973) *J Biol Chem* **248**: 3046.

S14. 12.00pm

Molecular Genetics of GM2-Gangliosidosis AB Variant: Deletion of a Single Base A_{410}, a Novel Mutation of the GM2-Activator Gene

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Glycosphingolipids in mammalian tissues are catabolized by the sequential release of monosaccharide units by specific lysosomal acid glycohydrolases. The catabolism of some of the glycosphingolipids by specific hydrolases requires the presence of activator proteins. One of these proteins is the GM2-activator which activates the lysosomal degradation of ganglioside GM2 by β-hexosaminidase A which consist of two subunits, α and β.

Inherited defects in any of the three protein components in the system, β-hexosaminidase subunits α and β, and the GM2 activator protein, may thus cause a GM2-gangliosidosis. Up to now, multiple genetic abnormalities in the α and β subunits of the β-hexosaminidase A have been identified. Mutations in the α-chain lead to Tay-Sachs disease, while mutations in the β-chain are responsible for the failure of both β-hexosaminidase A (αβ) and B (ββ) in Sandhoff's disease. GM2-Gangliosidosis due to a defective GM2-activator protein but with normal β-hexosaminidase isoenzym is known as the AB-variant.

Two mutations affecting the activator protein have been reported. Transitions of $T_{412} \rightarrow C$ and $G_{506} \rightarrow C$ lead to a deficiency of the GM2-activator in the lysosomes [1].

A novel mutation which will be reported here is a single base deletion A_{410} which leads to a frame shift. This results in the substitution of 33 aminoacids by other aminoacids and the loss of 24 aminoacids. Whereas the patient's fibroblasts produce apparently normal levels of activator mRNA, they lack a functional protein. No protein was detected by immunoprecipitation from cells treated with and without brefeldin A indicating premature degradation of the mutant GM2-activator in the ER. *In vitro* translation of mRNA in lysates without microsomes shows a smaller protein. We conclude that the instability of the mutant protein is responsible for the phenotype of the disease.

1. Sandhoff K, Harzer K, Fürst W (1995) In *The Metabolic and Molecular Basis of Inherited Diseases* (Scriver CR, Beaudet AL, Sly WS, Valle D, eds) 7th Edition. New York: Chapter 77, 2427–41. McGraw Hill.

S15. GLYCOSAMINOGLYCAN STRUCTURE AND BIOSYNTHESES

Supported by Seikagaku Corporation

Chairs: Ulf Lindahl, Vincent Hascall

S15. 9.50am

Proteoglycan Biosynthesis in a Madin-Darby Canine Kidney Strain II Cell Line Deficient in Transport of UDP-Galactose into the Golgi Complex

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[2]*Escola Paulista de Medicina, Sao Paolo, Brazil.*

Brandli *et al.* [1] previously isolated and characterized a mutant of Madin-Darby canine kidney strain II cell line resistant to *Ricinus communis* agglutinin. The biochemical defect leading to the altered phenotype was determined to be a 98% deficiency in the rate of transport of UDP-galactose into Golgi vesicles; transport of UDP-GlcNAc and CMP-sialic acid into these vesicles was similar to that of wild-type cells. The defect resulted in enrichment of cell surface glycoconjugates bearing terminal GlcNAc and of glucosylceramides.

Because galactose is a component of the linkage region of virtually all proteoglycans, we have examined their biosynthesis in the above cell line. Using radiolabelled sulfate and glucosamine we have found that both mutant and wild-type cells synthesize comparable amounts of chondroitin6-sulfate and heparan sulfate; however, the mutant cells synthesize significantly lower amounts of keratan sulfate. This suggests that the availability of nucleotide sugars in the Golgi lumen plays a selective role in the biosynthesis of proteoglycans.

1. Brandli *et al.* (1988) *J Biol Chem* **263**: 16283.

S15. 10.10am

Structural Variability of the Glycosaminoglycan-Protein Linkage Region

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In the early 80s we initiated our structural studies of the glycosaminoglycan-protein linkage region to re-evaluate the so-called common structure GlcA-Gal-Gal-Xyl-Ser in this critical part of the glycosaminoglycan biosynthetic pathway. The linkage region is first constructed in biosynthesis, and therefore possible structural differences in this region may influence that of the repeating disaccharide region to be synthesized thereafter. Sulfation of C4 of the third sugar residue Gal was first discovered in one of the four hexasaccharide-serines isolated from chondroitin 4-sulfate of rat chondrosarcoma [1]. Systematic structural studies for the next several years revealed some additional modifications such as C2 phosphorylation of Xyl or C6 sulfation of both Gal residues in some of the 13 hexasaccharides isolated from chondroitin 6-sulfate of shark cartilage [2, 3]. In contrast, no such sulfation of the Gal residues was found in heparin or heparan sulfate. More recently five linkage hexasaccharide alditols were isolated from dermatan sulfate of bovine aorta [4]. Two of the five compounds had the hitherto unreported hexasaccharide core with an internal IdoA residue; ΔHexA-GalNAc-IdoA-Gal-Gal-Xyl-ol. One is monosulfated on C4 of the GalNAc and the other is disulfated on C4 of the GalNAc and of the third sugar residue Gal. Thus, structural differences appear to exist in the linkage region among different species of sulfated glycosaminoglycans although it remains to be assessed whether these modifications are involved in the biosynthetic sorting mechanisms of different glycosaminoglycans. These structural studies have also clearly demonstrated that at least several structurally distinct subclass chains exist in each glycosaminoglycan species. In contrast, another recent study from our laboratory [5] detected only a single species of the novel linkage hexasaccharide structure ΔHexA-GalNAc-(4-sulfate)-GlcA-Gal(4-sulfate)-Gal-Xyl-ol in the chondroitin 4-sulfate chain of inter-α-trypsin inhibitor in human plasma. An overview and update on the structural variability of the glycosaminoglycan-protein linkage region will be presented.

1. Sugahara *et al.* (1988) *J Biol Chem* **263**: 10168.
2. Sugahara *et al.* (1992) *J Biol Chem* **267**: 6027.
3. de Waard *et al.* (1992) *J Biol Chem* **267**: 6036.
4. Sugahara *et al.* (1995) *J Biol Chem* in press.
5. Yamada *et al.* (1995) *Glycobiology* in press.

S15. 10.30am

Amino Acid Sequences Regulate Heparan Sulfate Assembly in Proteoglycans

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To study how cells regulate the synthesis of heparan sulfate chains on proteoglycans, we have examined the assembly of glycosaminoglycans on chimeric proteoglycans containing segments of betaglycan or syndecan-1 fused to protein A. Transient expression of the chimeras in Chinese hamster ovary cells showed that the site containing heparan sulfate in betaglycan consists of a Ser residue flanked by Trp and a cluster of acidic residues [1]. In syndecan-1, three sites near the N-terminus (-DG*SG*DDSDNF*SGSG*TG-) supported heparan sulfate synthesis. The strongest site contained the repeat unit -SGSG-. An unusual coupling phenomenon occurs across the adjacent SG dipeptides, leading to a greater proportion of heparan sulfate than predicted by the behavior of each site acting independently. A survey of the protein database revealed that most heparan sulfate proteoglycans contain repetitive $(\text{Ser-Gly})_n$ segments ($n \geqslant 2$) and a nearby cluster of acidic residues. Available data suggests that these control sequences interact with a key transferase that adds the first α-GlcNAc residue to nascent oligosaccharide chains [2]. This enzyme has been partially purified and characterized.

1. Zhang L, Esko JD (1994) *J Biol Chem* **269**: 19295.
2. Fritz TA, Gabb MG, Wei G, Esko JD (1994) *J Biol Chem* **269**: 28809.

S15. 10.50am

Formation of Chondroitin Sulfate and Heparan Sulfate on Recombinant Domain I of Mouse Perlecan

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Domain I of mouse perlecan was expressed in Chinese hamster ovary (CHO K1) cells in order to investigate the role of core protein in selective addition of chondroitin sulfate or heparan sulfate to proteoglycan. Gel chromatography followed by characterization of glycosaminoglycans by the use of glycosaminoglycan lyases showed that two populations of recombinant proteoglycans were synthesized. One population appeared to carry three short (10–15 kDa) heparan sulfate chains while the other population appeared to carry three similarly sized chondroitin/dermatan sulfate chains. There did not appear to be any domain I proteoglycans with heparan sulfate and chondroitin/dermatan sulfate on the same core protein molecule. These data demonstrate that domain I has functional sites for attachment of glycosaminoglycans and indicate that the glycosaminoglycan chains of native perlecan are probably grouped at its N-terminal end. This suggests that the likely function of domain I in perlecan would be to provide for the addition of glycosaminoglycan chains to the core protein. The formation of two distinct proteoglycan populations also indicates that the overall structure of the domain I core protein did not impede the action of enzymes for either heparan sulfate or chondroitin sulfate biosynthesis. Our results, in combination with data previously published concerning the synthesis of syndecan-1 and decorin by transfected CHO K1 cells, can be explained best by differential movement of individual core protein molecules to separate compartments for heparan sulfate and chondroitin/dermatan sulfate biosynthesis.

S15. 11.05am

Molecular Genetic Studies on the Biosynthesis of the *E. coli* K5 Capsule Polysaccharide: A Molecule Identical to *N*-Acetyl Heparosan

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The *E. coli* K5 polysaccharide, [-4)-β-D-GlcA-(1,4)-α-D-GlcNAc-(1-] is identical to *N*-acetyl heparosan, the first polymeric precursor of heparin. Studies in our laboratories have focused on the biosynthesis of this polysaccharide in *E. coli* and the mechanisms by which it is exported onto the cell surface. The entire nucleotide sequence of the K5 capsule gene cluster has been determined. This has revealed that eleven proteins are required for the expression of a K5 capsule on the cell surface. Seven of the encoded proteins are involved in the export of the polysaccharide across the inner and outer membranes whilst four proteins appear to be involved directly in the synthesis of the K5 polysaccharide. Of these four proteins, one, KfiD, is a UDP-Glc dehydrogenase which catalyses the formation of UDP-GlcA for incorporation into the K5 polysaccharide. A second, KfiC, is the transferase enzyme capable of chain elongation by the addition of alternate GlcA and GlcNAc at the non-reducing end of the polysaccharide. This may be comparable to heparin biosynthesis which is also believed to involve a single GlcA/GlcNAc transferase enzyme. Multiple alignments of the amino acid sequence of the KfiC protein with other glycosyl transferase enzymes identified three conserved domains. The functions of the other two proteins (KfiA and B) are less clearly understood but may be involved in the initiation of polysaccharide biosynthesis.

S15. 11.20am

Characterization of the two N-deacetylase/ N-sulfotransferase genes

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The charge density of heparan sulfate is established during its biosynthesis and a combined N-deacetylase/N-sulfotransferase enzyme has a key regulatory role in determining the final structure. Proteins expressing N-deacetylase/N-sulfotransferase activities have been purified and cloned from a mouse mastocytoma and from rat liver. The enzyme purified from the mouse mastocytoma is encoded by a 4 kb mRNA while the rat liver transcript contains 8.5 kb. Data from Southern blots indicate that the two enzymes are encoded by different genes.

To gain an insight into the regulation of gene expression of the two enzymes we have started to characterize the genomic organization of both enzymes in mouse. Most of the exon/intron organization of the gene corresponding to the 4 kb transcript has been elucidated and the organization of the other gene is currently being investigated. To determine the evolutionary relationship between the two genes, their exon arrangements will be compared. Structural characterization of the 5′ flanking region will provide the molecular basis for the understanding of the transcriptional control of the genes for these important enzymes.

S15. 11.40am

Biosynthesis of Syndecan-1 Heparan Sulfate Glycosaminoglycans in Human Myeloma Cells

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Heparan sulfates (HS) play a critical role in the regulation of both normal and pathological processes including tumor cell growth and metastasis. To better understand the cellular mechanisms that determine HS structure and function, we are studying several human myeloma B-cell lines known to have variable expression of the HS-proteoglycan, syndecan-1. To begin to identify and characterize HS biosynthetic enzymes in these cell lines, the photoaffinity analogs 5-azido-UDP-GlcUA, 5-azido-UDP-Glc and 2-azido-PAP were used. Using [^{32}P]5N_3UDP-GlcUA and [^{32}P]5N_3UDP-Glc, a microsomal pro-

tein of 74 kDa has been identified as a putative heparin UDPGlcUA/UDP-GlcNAc glycosyltransferase based on photoincorporation of both photoprobes. Photolabeling of the 74 kDa protein was Mn^{2+} dependent, saturable and inhibited by (best to last) UDP-GlcNAc, UDP-GlcUA, UDP-Glc and UDP-GalNAc. A correlation between syndecan-1 expression and photoincorporation levels was also observed, i.e. cells with no syndecan-1 expression had minimal labeling of the 74 kDa protein. Using $[^{32}P]2N_3PAP$, photolabeled proteins of 97 kDa and 60 kDa were observed, consistent with reported MWs of heparin sulfotransferases. The syndecan-1 expressing B-cells represent a model system for the study of the regulation of HS biosynthesis and syndecan function.

Supported by NIH RR-94-002 and Mizutani Foundation for Glycoscience.

S15. 11.55am

Termination of Aggrecan Chondroitin Sulfate – Effect of Sulfate Supply

A. Plaas[1], L. West[1], R. Midura[2] and V. Hascall[3]
[1]*Shriners Hospital, Tampa, FL, USA.*
[2]*University of Iowa, Iowa City, USA.*
[3]*Cleveland Clinic, Cleveland, USA.*

The role of chondroitin sulfate (CS) on aggrecan, in providing appropriate tissue hydration, is a function of the concentration of CS, the length of the chains and the arrangement of sulfate esters along the polymer backbone. There has also been much interest in the addition of specific components at the non-reducing terminal of the CS chain during biosynthesis. Such structures could serve as signals for chain termination and provide unique structural and immunological properties to the PG. To investigate the factors which determine the nature of CS terminal structures on calf epiphyseal cartilage aggrecan, such residues were isolated and quantitated by ion-exchange chromatography (AS4A IonPac, eluted with a gradient of 10–500 mM trifluoroacetate pH 7.0) of 2-aminopyridine-derivatized chondroitinase ACII digestion products. Analyses showed that about 70% of chains terminated with GalNAc-4S and about 20% terminated with GalNAc-4,6S. The remaining 10% were terminated with GlcUA, which was always adjacent to a sulfated GalNAc. When explant cultures of epiphyseal cartilage were radiolabelled with ^{3}H-glucosamine, the CS synthesised was essentially identical in size, sulfation pattern and terminal residue composition to that present in the tissue. When aggrecan synthesis was allowed to proceed under conditions of reduced sulfate supply (sulfate free medium or supplementation with 10 mM chlorate) the extent of sulfation of CS was reduced by 90% but the hydrodynamic size of the CS chains was essentially unaffected. This suggests that CS chain elongation is not critically controlled by concurrent sulfation. Analysis of the chain terminal residues showed that under these conditions about 70% of CS chains terminated with an unsulfated GalNAc and the remaining 30% terminated with GlcUA adjacent to an unsulfated GalNAc. These data indicate that for CS synthesis on aggrecan a hexosamine is the preferred terminal sugar, even in the absence of complete sulfation. However, lack of sulfation of this residue appears to allow addition of a GlcUA residue to a greater proportion of the elongated GAG chains, prior to secretion of aggrecan from the cell.

S15 POSTERS

S15

Studies on *O*-Sulfotransferase of Heparan Sulfate: Purification and Characterization of Heparan Sulfate 6-Sulfotransferase from the Culture Medium of CHO Cells

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O-sulfation at various positions of heparan sulfate is one of the important steps in determining the structure of each functional domain in heparan sulfate. We found that more than 90% of heparan sulfate 6-sulfotransferase activity in cultured CHO cells, which transfers sulfate to position 6 of *N*-sulfoglucosamine in heparan sulfate, was secreted into the serum-free culture medium, while 94% of heparan sulfate 2-sulfotransferase activity, which transfers sulfate to position 2 of the iduronic acid residue, was retained in the cell layer.

Heparan sulfate 6-sulfotransferase was purified 10 700-fold to apparent homogeneity with a 40% yield from the serum-free culture medium of CHO cells. The isolation procedure included affinity chromatography of the first heparin-Sepharose CL-6B (stepwise elution), 3′,5′-ADP-agarose, and the second heparin-Sepharose CL-6B (gradient elution). SDS-PAGE of the purified enzyme showed two protein bands with molecular masses of 52 kDa and 45 kDa. Both proteins appeared to be glycoproteins since their molecular masses decreased after *N*-glycanase digestion. When completely desulfated and *N*-resulfated heparin was used as acceptor, the purified enzyme transferred sulfate to position 6 of the *N*-sulfoglucosamine residue, but did not transfer sulfate to the amino group of the glucosamine residue or to position 2 of the iduronic acid residue. Heparan sulfate was also sulfated by the purified enzyme at position 6 of the *N*-sulfoglucosamine residue. These results suggest that 6-*O*-sulfation and 2-*O*-sulfation of heparan sulfate may be catalysed by different enzymes.

S15

Molecular Cloning and Expression of Chick Chondrocyte Chondroitin 6-Sulfotransferase

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Chondroitin 6-sulfotransferase (C6ST) catalyses the transfer of sulfate from 3′-phosphoadenosine 5′-phosphosulfate to position 6 of *N*-acetylgalactosamine residue of chondroitin. The enzyme has been previously purified to apparent homogeneity from the serum free culture medium of chick chondrocytes. We have now cloned the cDNA of the enzyme. This cDNA contains a single open reading frame that predicts a protein composed of 458 amino acid residues. The protein predicts a Type II transmembrane topology similar to other glycosyltransferases

and heparin/heparan sulfate *N*-sulfotransferase/*N*-deacetylases. Evidence that the predicted protein corresponds to the previously purified C6ST was the following: (a) the predicted sequence of the protein contains all of the known amino acid sequence; (b) when a cDNA containing the sequence coding for the C6ST was introduced in a eukaryotic expression vector and transfected in COS-7 cells, the enzyme activity was expressed 20-fold over controls; (c) polyclonal antibody raised against a fusion peptide, which contained 150 amino acid residues deduced from the nucleotide sequence of the cDNA, cross-reacted to the purified C6ST; and (d) the predicted protein contained six potential sites for N-linked sugar chains, which fits with the glycoprotein nature of C6ST. The amino-terminal amino acid sequence of the purified protein was found in the transmembrane domain, suggesting that the purified protein might be released from the chondrocytes after proteolytic cleavage in the transmembrane domain.

S15

Defructosylated Capsular Polysaccharide from *Escherichia coli* Strain K4 can be Converted into Dermatan by a Glucuronyl-C5-Epimerase

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Capsular polysaccharide from *E. coli strain* K4 is composed of a chondroitin backbone ([D-GlcAβ1,3-D-GalNAcβ1,4]$_n$) with β-fructofuranose units linked to C3 of the GlcA residues. Dermatan ([L-IdoAa1,3-D-GalNAcβ1,4]$_n$) and defructosylated K4 were incubated with microsomes from cultured human skin fibroblasts in the presence of ^3H_2O, and the kinetics of the GlcA C5-epimerase were determined by measuring the rate of ^{3}H incorporation at C5 of hexuronic acid units. K_m was similar for both substrates, but V_{max} was >3 times higher for dermatan; the reaction is freely reversible. The distribution of GlcA and IdoA units in epimerized products, as assessed by size-determination of chondroitinase ACI-resistant fragments, suggested a processive rather than completely random mode of action of the epimerase. Identification of disaccharides generated by *N*-deacetylation (hydrazinolysis) followed by deaminative cleavage, indicated that equilibrium was reached at an IdoA:GlcA ratio of 15:85. The higher contents of IdoA found in dermatan sulfate are presumably due to coupling between the epimerization and sulfation reactions.

S15

GalNAc Transfer to the Common Carbohydrate-Protein Linkage Region of Sulfated Glycosaminoglycans. Identification of UDP-GalNAc: GlcA-Oligosaccharide α-*N*-Acetylgalactosaminyltransferase in Bovine Serum

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During the course of a study to elucidate the role of modification [1] of the common polysaccharide-protein linkage structure of glycosaminoglycan, GlcAβ1,3Galβ1,3Galβ1,4Xylβ1-*O*-Ser, in biosynthetic sorting mechanisms of the different glycosaminoglycan chains, a novel GalNAc transferase was discovered in bovine serum. The enzyme catalysed the transfer of [^{3}H]GalNAc from UDP-[^{3}H]GalNAc to linkage tetrasaccharide- and hexasaccharide-serines [2] and to various regular oligosaccharides containing terminal GlcA, which were prepared from chondroitin and chondroitin sulfate using testicular hyaluronidase digestion. The labelled products obtained with the linkage tetra- and hexasaccharide-serines and with the tetrasaccharide (GlcAβ1,3GalNAc)$_2$ were resistant to chondroitinase AC II and β-*N*-acetylhexosaminidase, but sensitive to digestion with α-*N*-acetylgalactosaminidase, indicating that the enzyme is an α-*N*-acetylgalactosaminyltransferase. This finding is in contrast to that of Rohrmann *et al.* [3], who reported that a corresponding product was susceptible to digestion with β-*N*-acetylhexosaminidase. The presence of a sulfate group at C4 of the penultimate GalNAc or Gal units markedly inhibited the transfer of GalNAc to the terminal GlcA, while a sulfate group at C6 of the GalNAc had little effect on the transfer. Moreover, a slight but significant transfer of [^{3}H]GalNAc was observed to an oligosaccharide containing terminal 2-*O*-sulfated GlcA as acceptor, whereas no incorporation was detected into oligosaccharides containing terminal unsaturated or 3-*O*-sulfated GlcA units. These results suggest that this novel serum enzyme is a UDP-GalNAc: GlcA-oligosaccharide α1,3- or 1,4-*N*-acetylgalactosaminyltransferase. A relationship of this enzyme to galactosaminoglycan biosynthesis is presently unclear.

1. Sugahara K *et al.* (1988) *J Biol Chem* **263**: 10168–74.
2. Goto F, Ogawa T (1993) *Pure Appl Chem* **65**: 793–801.
3. Rohrmann K *et al.* (1985) *Eur J Biochem* **148**: 463–69.

S15

Purification of the Protein Catalysing both GlcNAc and GlcA Transfer in the Chain Elongation Reaction of Heparin/Heparan Sulfate

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The biosynthesis of heparin/heparan sulfate is initiated by the formation of polysaccharide chains, composed of alternating GlcNAc and GlcA units. The GlcNAc and GlcA transferase activities catalysing the polymerization reaction can be assayed by measuring the transfer of radiolabelled monosaccharide units from the appropriate UDP-sugars to exogenous oligosaccharide acceptors. Such acceptors were derived from *E. Coli* K5 capsular polysaccharide which has the same repeating disaccharide unit as the heparin/heparan sulfate precursor polysaccharide.

We have identified a 70 kDa protein from bovine serum catalysing both GlcNAc and GlcA transferase activities. The two activities are inseparable even on SDS-PAGE [1]. Purification on a larger scale of the GlcNAc and GlcA transferases from bovine serum through consecutive runs on Red-Sephar-

ose, ConA-Sepharose, Phenyl-Sepharose, Superdex 200, UDP-hexanolamine-agarose, Mono Q followed by chromatofocusing also showed that the two activities appeared together in all steps. This procedure gives an apparent ~200 000-fold increase in specific activities of both GlcNAc and GlcA transferases. SDS-PAGE of active protein fractions from the last two steps in the purification procedure showed a silver staining band along with the enzyme activities that correspond to an apparent M_r of 70 000. The amount of prepared protein was sufficient for amino acid sequence analysis.

1. Lind T, Lindahl U, Lidholt K (1993) *J Biol Chem* **268**: 20705–8.

TUESDAY 22 AUGUST, AFTERNOON

PLENARY LECTURES

S16. 2.10pm

Regulation of Glycosyltransferase Gene Expression: What Have We Learned From Studies On The Murine β4-Galactosyltransferase Gene

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The glycosylation phenotype of a cell results in part, from the regulated expression of individual glycosyltransferase genes. Although many of the glycosyltransferases are constitutively expressed, it is becoming increasingly clear that cell-specific mechanisms of expression are also involved in the transcriptional regulation of these genes. However, the nature of the DNA sequence elements and protein factors required for both ubiquitous and cell-specific glycosyltransferase gene expression are poorly understood.

β4-Galactosyltransferase participates in glycan biosynthesis (all cells) and lactose biosynthesis (mammary gland). Our studies show that complex control mechanisms are utilized in the transcriptional regulation of this gene. To date, three promoter regions have been identified: (i) a male germ cell-specific promoter that functions exclusively during the later stages of spermatogenesis; (ii) a ubiquitous promoter that functions in all cells; and (iii) a mammary cell-specific promoter that functions primarily in the late pregnant and lactating mammary gland. The net result of the use of the different promoters is the production of transcripts with a 5′-untranslated region of 730 bp (male germ cells), ~200 bp (all cells), or ~25 bp (lactating mammary gland). It is known that the synthesis rate of a protein is influenced not only by the steady state levels of its mRNA but also by the structure/length of the 5′-end of the mRNA. This additional level of control appears to have been exploited by mammals to generate the elevated levels of β4-GT required by the lactating mammary gland for lactose biosynthesis. A switch from the ubiquitous to the mammary cell-specific promoter in this tissue results not only in the production of increased levels of mRNA, but the mRNA synthesized is predicted to be more efficiently translated relative to its ubiquitous counterpart.

S17. 2.40pm

Syndecans and Glypicans: Cell Surface Proteoglycans that Regulate Cell Behaviour

M. Bernfield
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Cell surface proteoglycans employ economical strategies to exert their effects on cells. Core protein domains place the glycosaminoglycan (GAG) chains, usually heparan sulfate, at the cell surface where they can bind a variety of protein ligands, including growth factors, cytokines, extracellular matrix components, proteases and anti-proteases. Glypicans are linked to membrane phosphatidylinositol and contain cysteine-rich presumably globular proteins which can be shed by phospholipases. Cerebroglycan is restricted to the brain while glypican is ubiquitous. Syndecans are transmembrane proteins containing short cytoplasmic domains and extended extracellular domains which can be shed intact by proteases. The syndecans have been conserved for over 640 million years, and in mammals are represented by four gene products. They are autocrine at the cell surface, acting as matrix anchors and coreceptors in concert with signal transducing receptors, or paracrine when shed, acting to stimulate or inhibit cell growth depending on the growth factor and on the nature of the heparan sulfate. Loss of syndecan-1 causes epithelial cells to acquire a mesenchymal cell phenotype and anchorage independent growth. During embryogenesis, syndecans are expressed very early, are induced in morphogenetic, rather than histological patterns, and become predominant in distinct tissues while they acquire cell-type specific heparan sulfate chains. During the repair of skin wounds, they are induced in dermal mesenchymal cells only while these cells orchestrate the repair. This induction is due to a neutrophil-derived antibacterial peptide which may augment wound repair by both inducing syndecans and killing bacteria. Thus, a variety of mechanisms control cell surface proteoglycans, which in turn, regulate cell behaviour.

S18. 3.10pm

Assessing Glycosyltransferase Function *In Vivo* Using Transgenic, Gene-Targeting and Tissue-Specific Approaches

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Experimentally-induced gene inactivation in embryonic stem cells has been previously used to ablate *mgat-1*-encoded GlcNAc-TI function and complex N-linked oligosaccharide production *in vivo* in the mouse. Results demonstrated that embryonically derived complex N-glycans were crucial for post-implantation development, especially in morphogenic processes. While mutant embryos developed into embryonic day

(E) 9, we have found that parentally-derived complex N-glycans appear to be available prior to the induction of embryonic *mgat-1* gene expression between E7 and E9. Unlike pre-implantation development, during which embryos contained complex N-glycans regardless of embryonic *mgat-1* inactivation, only vesicles within extraembryonic endodermal cells in post-implantation *mgat-1*-null embryos invariably exhibited binding to the lectin phytohemagglutinin. These observations indicate that perhaps a subset of the maternal complex N-linked oligosaccharide repertoire may be required to at least E9 when embryonic *mgat-1* is induced and required. To dissect the roles of oligosaccharide structures in mammalian embryogenesis, both *mgat-1* and the gene encoding GalNAc transfer function to serine and threonine residues (GalNAc(thr/ser)-TI) have been mutated using the Cre recombinase-loxP gene-targeting approach. We have produced homozygous mutations resulting in either systemic or conditional loss of GalNAc(thr/ser) TI function. Viable mice comprising the conditional model were found to have undergone quantitative GalNAc(thr/ser)-TI gene mutation specifically in thymocytes. We will detail current findings regarding enzymatic, structural, and ontogenic phenotypes.

S16. GLYCOSYLATION REGULATION THROUGH GLYCOSYLTRANSFERASE GENES

Chairs: Harry Schachter, Naoyuki Taniguchi

S16. 4.00pm

Suppression of Lung Metastasis of Melanoma B16-F1 by *N*-Acetylglucosaminyltransferase III Gene Transfection

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The β1-6 structure of *N*-oligosaccharides, catalysed by β1-6 *N*-acetylglucosaminyltransferase (GnT-V), is associated with metastatic potential. Transfection of the gene for β1-4 *N*-acetylglucosaminyltransferase (GnT-III) into B16F1 murine melanoma cells reduced the level of β1-6 structure by competing for substrate. This resulted in the suppression of lung metastasis in syngeneic and nude mice, decreased invasiveness into matrigel, inhibition of cell attachment to collagen and laminin, and suppressed collagenase activity *in vitro*. The gene transfection altered the *N*-oligosaccharides of lysosome membrane glycoproteins, which could disrupt lysosomal proteases responsible for invasion. Moreover, an elevated amount of E-cadherin with increased bisected *N*-oligosaccharides was observed at the cell-cell border, which could be suppressive for metastasis. Our results directly demonstrated a causative role for β1-6 branches in metastasis.

S16. 4.20pm

Molecular Cloning of Sialyltransferases

S16. 4.35pm

Characterization of a Mouse B16 Melanoma Mutant Deficient in Glycosphingolipids

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[2]*Division of Physiology and Pathology, National Institute of Radiological Science, 4-9-1 Anagawa, Chiba-shi, Chiba 260, Japan.*

Mouse B16 melanoma cell line GM-95 (MEC-4), primarily isolated as a GM3-negative mutant, was shown to lack all the glycosphingolipids. The content of ceramide in the mutant, however, was normal. The deficiency of glycosphingolipids was attributed to the first glycosylation step since no glucosylceramide synthase activity was detected. In contrast, there was no significant differences in GM3 synthase activity between the parental (MEB-4) and mutant cells. Glycoprotein synthesis was not affected by the mutation because the lectin blot analyses using Con A and wheat germ lectin as probes showed no significant differences between B16 and GM-95 cells.

GM-95 cells exhibited an elongated fibroblastic morphology and parallel arrangement, whereas MEB-4 cells showed irregular shapes and tend to overlap each other. The growth rate of GM-95 cells were reduced significantly compared to MEB-4 cells. The population doubling times of GM-95 and MEB-4 cells were 28 h and 19 h, respectively. GM-95 cells, however, could be maintained in serum free defined medium for 2 months. The result suggests glycosphingolipids are not indispensable for the survival and proliferation of B16 cells.

GM-95 cells would provide ideal tools for the study of glycosphingolipids.

S16. 4.50pm

Elimination of the Major Porcine Xenoantigen, Galα(1,3)Gal, by Expression of α(1,2)Fucosyltransferase

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The major barrier for xenotransplantation of pig organs to humans is the presence of natural human antibodies to the major porcine epitope Galα(1,3)Gal which cause hyperacute graft rejection (HAR) of vascularized organs within a few minutes. Surprisingly, almost all the human natural antibodies (both IgM and IgG) react with Galα(1,3)Gal epitopes which are found on many glycosylated cell surface molecules of the pig. There are several possibilities for overcoming HAR and here we show that expression of H substance eliminates the expression of Galα(1,3)Gal. We have shown that there is a

hierarchy in the action of glycosyltransferases that are simultaneously expressed within the same cell and that H transferase overrides that of the Gal transferase. COS cells simultaneously transfected with cDNA clones encoding α1,2fucosyltransferase and α1,3galactosyltransferase show preferential expression of the H substance (synthesised by the α1,2fucosyltransferase) rather than Galα(1,3)Gal (synthesised by the α1,3galactosyltransferase). In a pig kidney cell line, which expresses both the Galα(1,3)Gal and H, the increased expression of H, induced by the transfection and stable expression of α1,2fucosyltransferase, resulted in a major decrease in expression of Galα(1,3)Gal. Coexpression of the α1,2fucosyltransferase and the α1,3galactosyltransferase in either COS cells or the pig cell line, resulted in a major decrease in human antibody binding and in complement mediated cell lysis. The findings have important implications for xenotransplantation in that α1,2fucosyltransferase transgenic pigs could be a source of donors for xenotransplantation to humans.

S16. 5.05pm

Regulation of *N*-Acetylglucosaminyltransferase V and Expression of its Cell Surface Oligosaccharide Products

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[2] *Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA, USA.*

The expression of oligosaccharides containing [GlcNAcβ(1,6)-Man], the 'β(1,6) branch', on cell surface glycoproteins is now known to be regulated by several mechanisms. This branch is exclusively synthesized by the glycosyltransferase GlcNAc-T V, whose cDNA was recently isolated. Increased expression of GlcNAc-T V and its cell surface products have been observed in cells which are mitotically active and migratory. The oncogenes, *neu/her*-2, *src*, and *ras*, cause a specific, significant increase in both GlcNAc-T V mRNA and enzymatic activity, as well as increased cell surface β(1,6) branched N-linked oligosaccharides. In the case of *src* expression, its effects on GlcNAc-T V can be inhibited by herbimycin-A, a *src*-family tyrosine kinase inhibitor and by a temperature-sensitive mutation in its kinase activity. *Neu/her*-2, *src*, and *ras* all feed into the MAPK pathway via the Raf-1 kinase. A dominant-negative mutant Raf-1 (Raf C4B) is able to inhibit signals propagated from these oncogenes to the Mitogen Activated Protein Kinase Kinase and will allow us to determine if GlcNAc-T V gene expression is regulated by this pathway.

In the systems investigated to date, GlcNAc-T V is upregulated in many transformed cells, especially in metastatic sublines. The B-16 mouse melanoma cell line is a highly invasive tumour line which has the highest GlcNAc-T V specific activity reported thus far, 2.5 nmol h^{-1} mg. Towards understanding the function of β(1,6) branches during cell adhesion and metastasis, we have constructed mutants of the B-16 cell line that have reduced GlcNAc-T V enzyme activity and cell surface products by the expression of a GlcNAc-T V antisense cDNA. These cells have also been stably transfected with the firefly luciferase gene, and produce light in a standard luciferase assay. This will allow us to quantitate cell adhesion and metastatic potential by measuring luciferase activity.

S16. 5.20pm

Studies on the Biosynthesis of N-Glycoprotein Glycans in the Connective Tissue of the Freshwater Snail *Lymnaea stagnalis*

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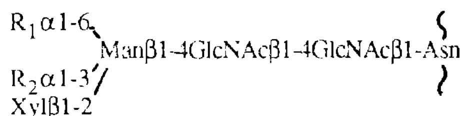
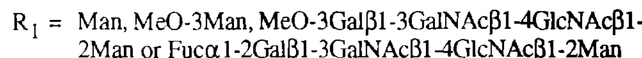
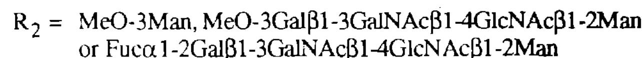
It has been suggested that the pore cells in the connective tissue of the freshwater snail *Lymnaea stagnalis* synthesize and store hemocyanin, a high-molecular-mass copper-containing oxygen-transport protein. Hemocyanin is a glycoprotein with a carbohydrate content of 3% (by mass). Previously, in our group the primary structures of the carbohydrate chains have been identified, demonstrating a series of novel N-linked glycans, which can be summarized as follows:







In this paper a pathway will be presented, discussing the biosynthesis of the unusual antennary elements of the hemocyanin glycans. To this end a series of enzymatic studies have been carried out focused on transferases detected in connective tissue and probably involved in this biosynthesis, namely, β2GlcNAc-T I, β2GlcNAc-T II, β2Xyl-T, β4GalNAc-T, β3Gal-T and α2Fuc-T. Several relevant carbohydrate chains, varying from monosaccharides to glycoproteins, were tested as suitable acceptors.

S16. 5.35pm

The Expression of Oligosaccharyltransferase (48 kDa) during Differentiation of P19 EC Cells Induced by Retinoic Acid and the Relation with Carbohydrate Deficient Glycoprotein Syndrome

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Oligosaccharyltransferase (OST) catalyses the transfer of oligosaccharide chains from dolichylpyrophosphoryl oligosaccharide to the asparagine side chain of Asn-X-Ser/Thr sites. We have cloned mouse and human cDNA of OST as well as human genomic OST. P19 EC cells differentiate multipotentially into neuronal cells, cardiac cells and skeletal muscle cells depending on the concentration of retinoic acid. We prepared the differentiation-specific cDNA library by subtracting the mRNA of the undifferentiated cells from that of the differentiated cells. During the DNA sequence analysis of each of the clones from the differentiation-specific library, we found the cDNA clone (mOST-1) that was highly homologous to yeast WTB1 and dog

oligosacchryltransferase (48 kDa) cDNA. The expression of mOST-1 was induced during the differentiation of P19 EC cells by retinoic acid over 12–24 h. mOST-1 was expressed in various tissues including lung, brain and liver of mouse embryos. It did not show developmental changes in these tissues. We obtained human OST-1 (hOST-1) cDNA from a fetal brain cDNA library and human genomic OST-1 from a cosmid library in order to examine the relation between the expression of OST-1 and Carbohydrate Deficient Glycoprotein Syndrome in which glycosylation of asparagine-linked oligosaccharides was affected.

S16. 5.50pm

Transcriptional Regulation of β-Galactoside α2,6-Sialyltransferase in B Lymphocytes

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A single gene, *SIAT1*, encodes the β-galactoside α2,6-sialyltransferase from which a diverse array of mRNA isotypes, most of which differ from each other only in the 5′ untranslated region, are expressed in a developmental-, cell-, and tissue-specific manner. 5′ untranslated leader heterogeneity results from differential usage of physically distinct and tissue specific promoters for transcriptional initiation. Earlier work documented glucocorticoid responsive transcription of *SIAT1* from a promoter region active only in adult hepatocytes and in the small intestinal epithelium of pre-weaning animals.

Developmental maturation of B lymphocytes is accompanied by dramatic elevation of *SIAT1* expression and appearance of cell surface α2,6-sialic acid that are requisite for recognition by the cell adhesion molecule, CD22. Induced *SIAT1* expression results by transcription from a novel promoter. In order to characterize this unique regulatory region, the transcription initiation site of B cell-specific *SIAT1* mRNA was mapped by combined S_1 nuclease protection and 5′-RACE analysis. Genomic region 5′ of the transcriptional initiation site contains putative consensus sequences for the binding of NF-IL6, NFκB, AP-1, and C/EBP. *CAT* reporter genes under the control of serially truncated segments of the 5′-flanking B-specific *SIAT1* region were constructed. The results suggest that the 740 bp segment residing immediately 5′ of the transcriptional initiation site is necessary and sufficient for high level expression of *CAT* in transiently transfected Louckes, a human mature B cell line. Furthermore, only minimal expression was observed in parallel transfections of the same constructs into HepG2, a human hepatic derived cell line. Requisite cis elements for transcription and cellular specificity were further assessed by gel shift and DNase protection analysis.

S16. 6.05pm

Carbohydrate-Deficient Glycoprotein Syndrome Type II. An Autosomal Recessive *N*-Acetylglucosaminyltransferase II Deficiency Different From Typical Hereditary Erythroblastic Multinuclearity With A Positive Acidified Serum Lysis Test (HEMPAS)

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Carbohydrate-Deficient Glycoprotein Syndrome (CDGS) type II is a multisystemic congenital disease with underglycosylated glycoproteins suggesting defective N-glycan assembly. Fibroblast extracts from two patients with CDGS type II have a reduction of over 98% in UDP-GlcNAc:α-6-D-mannoside β-1,2-GlcNAc-transferase II (GnT II) [1]. We show that mononuclear cell extracts from one of these CDGS type II patients have no detectable GnT II activity and that similar extracts from eleven blood relatives of the patient have GnT II levels at 33 to 68% of normal consistent with an autosomal recessive disease. Similar extracts from a HEMPAS patient have an 89% reduction in GnT II [2]. However, CDGS type II erythrocytes show neither the serology nor the band 3 poly-*N*-acetyllactosamine patterns that are typical of HEMPAS.

Support by MRC of Canada.

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S16 POSTERS

S16

β-*N*-Acetylglucosaminidase in Insect Cells is Probably Involved in N-glycan Processing

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The β-*N*-acetylglucosaminidase activity in the lepidopteran insect cell lines Sf21 and Bm-N hydrolysed 4-nitrophenyl β-*N*-acetylglucosaminide, 4-nitrophenyl β-*N*-acetylgalactosaminide, tri-*N*-acetylchitotriose and an N-linked biantennary agalacto oligosaccharide. However, from the biantennary substrate, the β-*N*-acetylglucosaminidase exclusively removed β-*N*-acetylglucosamine from the α1,3-antenna. $GlcNAcMan_5GlcNAc_2$, the primary product of GlcNAc-transferase I, was not perceptibly hydrolysed. Ultracentrifugation indicated the insect cell β-*N*-acetylglucosaminidase exists in a soluble and a membrane-bound form. This latter form accounted for two thirds of the total activity and was associated with vesicles of the same density as those containing GlcNAc-transferase I. In contrast, β-*N*-acetylglucosaminidase activities from rat or frog (*Xenopus laevis*) liver and from mung bean seedlings were neither membrane-bound nor did they exhibit a strict branch specificity.

Insect cell derived, natural or recombinant glycoproteins frequently carry N-glycans of the structure $Man_3GlcNAc(Fuc)GlcNAc$. However, both fucosyltransferase and α-mannosidase II from insect cells have been shown to rely on the presence of a terminal GlcNAc-residue on the α1,3-arm. We suppose that the terminal GlcNAc residue transferred by GlcNAc-transferase I is removed by the unusual, membrane-bound β-*N*-acetylglucosaminidase after the action of α-mannosidase II and fucosyltransferase(s).

S16

A Novel UDP-GlcNAc:GlcNAcβ-R β1→4-*N*-Acetylglucosaminyltransferase from *Lymnaea stagnalis* with Sequence Similarity to Mammalian β1→4-Galactosyltransferases

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A cDNA encoding a novel glycosyltransferase, that may be involved in a variant pathway for the synthesis of complex type oligosaccharide chains, was cloned from the pond snail *Lymnaea stagnalis*. By heterologous hybridization, using bovine β1→4-galactosyltransferase cDNA as probe, a genomic clone from a snail library was isolated. This genomic clone was subsequently used to clone the corresponding cDNA from a prostate gland library. The isolated cDNA encodes a polypeptide of 490 amino acids with a type II membrane protein topology typical of glycosyltransferases. The C-terminal part, encoding the putative catalytic domain, shows considerable sequence similarity with the corresponding region of mammalian β1→4-galactosyltransferases, suggesting an evolutionary relationship. Expression of this cDNA in COS-cells and insect-cells revealed that the encoded enzyme transfers GlcNAc, rather than Gal or GalNAc, from the corresponding nucleotide-sugars to several β-*N*-acetylglucosaminides. Structural characterization by ^{1}H-NMR spectroscopy of products formed *in vitro* demonstrated that the enzyme can be identified as a UDP-GlcNAc:GlcNAcβ-R β1→4-*N*-acetylglucosaminyltransferase [1]. Acceptor specificity studies revealed that the *Lymnaea stagnalis* β1→4GlcNAcT is not a chitin synthase but is capable of using oligosaccharide structures that are found on glycoproteins and glycolipids. The enzyme prefers a GlcNAc that is β1→6 linked towards the next sugar. A new family of glycosyltransferases has hereby been discovered, consisting of enzymes that act on acceptor substrates with a terminal β-linked GlcNAc residue, establish a β1→4-linkage, but have a different nucleotide-sugar requirement.

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S16

Characterization of Two Members of a UDP-GalNAc Peptide *N*-Acetylgalactosaminyltransferase Family

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Mucin type O-linked glycosylation of threonine and serine amino acid residues is controlled by a family of unknown size of UDP-GalNAc:polypeptide *N*-acetylgalactosaminyl transferases. Originally Homa *et al.* [1] purified and cDNA cloned a bovine GalNAc-transferase, and based on sequence similarity the human counterpart has been cloned and sequenced. A second GalNAc-transferase was recently purified from human placenta, and cDNA cloning and sequencing revealed a homologous gene with 44% sequence similarity at the amino acid level. The two GalNAc-transferase genes have thus been termed GalNAc-T1 and GalNAc-T2, respectively. The human versions of GalNAc-T1 and -T2 have been expressed as soluble proteins corresponding to the originally purified soluble proteins in the Baculovirus system. Preliminary analysis of substrate specificity using a panel of synthetic acceptor substrate peptides revealed differences in substrate specificity. The genomic structure of the coding region of GalNAc-T1 and -T2 was determined from isolated P1 phage clones. The entire coding sequence of GalNAc-T1 was identified from a single P1 clone, whereas two non-overlapping P1 clones covered nearly the entire coding sequence of GalNAc-T2. A total of 10 introns were mapped in GalNAc-T1 and 13 in GalNAc-T2 and two of the intron/exon boundaries were similar in the two genes. Fluorescence *in situ* hybridization using the P1 clones showed that GalNAc-T1 localizes to chromosome 18q12-21, whereas GalNAc-T2 localizes to chromosome 1q4.1-4.2. The data showed that a least two polypeptide GalNAc-transferases with different substrate specificity are involved in mucin-type O-linked glycosylation.

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S16

Estrogen-induced Changes in Expression of Le^{x} on α_1-Acid Glycoprotein in Females and Transsexual Males

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Various glycoforms of α_1-acid glycoprotein (AGP) can be distinguished in human sera. They all contain five N-linked glycans, but they differ in the presence of di-, tri-, and/or tetra-antennary glycans and in the degree of fucosylation. The relative proportions of the AGP glycoforms are dependent on the (patho)physiological state. Changes in glycosylation of AGP originate from the liver and can be induced by several mediators like cytokines and hormones. It is known that use of estrogen for treatment of prostatic cancer in males induces a decrease in the diantennary glycan content of serum glycoproteins. In this study we examined the effect of estrogen on the expression of Le^{x} on AGP. Women using oral contraceptives showed a significant decrease in Le^{x} expression on AGP compared to women using no oral contraceptives. The same phenomenon was found in transsexual males and (post)menopausal women treated with estrogen. It can be concluded that estrogen has an inhibitory effect on the hepatic fucosylation of AGP in males and females.

S16

Isolation of Human Placenta α-1,2 Mannosidase IB cDNA

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Previously, mouse α-1,2 mannosidase IA [1] and mouse α-1,2 mannosidase IB [2] cDNAs were characterized and found to be 64% identical. Degenerate primers derived from conserved sequences in α-1,2 mannosidases [2] were used to amplify human placenta cDNA by the polymerase chain reaction and two products with 70% amino acid identity were obtained. The

first was found to correspond to the human cDNA isolated by Bause *et al.* [3], while the second was found to have about 90% amino acid identity with the corresponding region of mouse α-1,2 mannosidase IB. Using this second product as a probe, a human placenta lambda gt11 cDNA library was screened and a novel cDNA was isolated. This human α-1,2 mannosidase IB cDNA was found to contain sequences highly conserved among the class I α-1,2 mannosidases. Northern blot analysis of human tissues revealed a major transcript of 9.5 kb. These results indicate that there are at least two distinct human α-1,2 mannosidase cDNAs.

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S16

Retinoic Acid Induction of Murine UDPgalactose: β-D-galactosyl-1,4-*N*-acetyl-D-glucosaminide α-1,3-galactosyltransferase: Stimulation of Transcript Levels, Enzyme Activity, and Enzyme Secretion

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The enzyme UDP-Gal:β-D-Gal-α1,3-galactosyltransferase, (α1,3)GT, is induced upon treatment of the murine embryonal carcinoma cell line F9 with all-trans retinoic acid (RA). Northern blot analysis showed that RA-treatment for three days resulted in an eight-fold increase in steady-state levels of (α1,3)GT mRNA and this increase was slightly enhanced by treatment with dibutyryl cyclic AMP. Nuclear run-on assays demonstrated that the RA-mediated regulation was determined at least in part at the transcriptional level. Western blotting using *Griffonia simplicifolia*-I(GSI-B_4), revealed that F9 differentiation results in increases in the expression of terminal α-linked galactose in cellular glycoproteins. The time course of induction was also accompanied by significant increases in (α1,3)GT in cell culture media. This secretion may be a common biological process. Using a new solid-phase assay for the activity of (α1,3)GT, we were able to detect the enzyme in the sera of animals which have been previously reported to express (α1,3)GT, but activity was absent in human and baboon sera. In addition, the (α1,3)GT activity was present in media of several cell lines known to express the enzyme.

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S16

Expression of GDP-Fuc:Galβ1→4GlcNAc-R α1,3-fucosyltransferase V (Fuc-TV) Constructs in Insect Cells as Soluble, Secreted Enzymes

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The α1,3-fucosyltransferases (Fuc-Ts) form a family of enzymes related in their primary sequence, but differing in substrate requirements. We have studied the acceptor specificity of Fuc-TV. Using a Fuc-TV cDNA as template, DNA fragments were prepared by PCR that encode truncated forms of Fuc-TV lacking the transmembrane domain and part of the stem region. Expression of these constructs in insect cells using the baculovirus system resulted in the secretion of soluble, enzymatically active forms of Fuc-TV, starting at amino acid 31 and 62, respectively. In addition, to facilitate purification of these enzymes from the growth medium, Fuc-TV was expressed as a chimeric protein in which a portion of protein A was attached to the N-terminus of the enzyme, and in a His_6-tagged form. Strategies for the purification of the various Fuc-T constructs were developed. The molecular weight on SDS-PAGE of the short form appeared to be 41 kDa and of the longer form 44 kDa. The influence of different affinity-tails on the catalytic properties was determined by evaluating the acceptor specificity of the various constructs, using a panel of lipid-linked oligosaccharides, and compared to the specificity of the corresponding native membrane bound Fuc-TV.

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S16

Discovery of α1-4 Fucosyltransferase in Human Serum and Measurement of Activity in Cell Extracts and Body Fluids Using a New Solid-Phase Technique

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GDPFuc:Galβ1-3GlcNAc (Fuc to GlcNAc) α1-4 fucosyltransferase (α1-4FT) generates the Lewis a blood group antigen (Le^a) (Galβ1-3[Fucα1-4]GlcNAc-R) from the acceptor Galβ1-3GlcNAc-R. To study expression of this activity, we developed a novel solid-phase assay. Lacto-N-tetraose (LNT, Galβ1-3GlcNAcβ1-3Galβ1-4Glc) was chemically conjugated to bovine serum albumin (BSA) to generate the neoglycoprotein LNT-BSA. The Le^a product of the reaction was detected with a monoclonal IgG antibody (BG-5). The amount of BG-5 bound to product was quantified by second antibodies coupled to the recombinant bioluminescent protein aequorin. The α1-4FT assay was linear with respect to time and extract added, and was dependent on GDPFuc and LNT-BSA. We have measured α1-4FT activity in human serum and saliva, and in extracts of the human colon carcinoma cell line SW1116. Activity was absent, however, in extracts of other cell lines, such as human HL60 and murine F9 teratocarcinoma. This new assay may be useful in assessing the Lewis blood group status of individuals.

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S16

Comparison of the Expression of Cell Surface Poly-*N*-Acetyllactosamine-type Oligosaccharides in PC12 Cells with Those in its Variant PC12D

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PC12D cells, a new subline of PC12 pheochromocytoma cells, extend neurites faster than PC12 cells, responding not only to nerve growth factor (NGF) but also to cyclic AMP. PC12D cells also differ morphologically from PC12 cells, being flat in shape and having extended short processes without any stimulation.

We have shown that the amount of ^{3}H-glucosamine incorporated into membrane glycoproteins differed significantly between PC12 and PC12D, and the NGF-stimulation caused marked decrease in the oligosaccharides with high molecular weight, while PC12D cells were insensitive in this regard.

The oligosaccharides of interest were characterized by the presence of up to six or more disaccharide repeating units having structure (Gal β1-4GlcNAc β1-3)$_n$ which were linked to a conventional complex oligosaccharide core.

The poly-*N*-acetyllactosamine content in N-linked oligosaccharides of PC12 and PC12D cells correlated with changes in UDP-GlcNAc:Gal β1-4GlcNAc-R β3-*N*-acetylglucosaminyltransferase (GnT-i) activity, whereas UDP-GlcNAc:α6Man β6-*N*-acetylglucosaminyltransferase (GnT-V) and β1-4galactosyltransferase activities remained unchanged. This suggested that GnT-i catalyses a rate-limiting reaction in the expression of poly-*N*-acetyllactosamine in N-linked oligosaccharides of PC12 and PC12D cells. Thus, it appears that the content of poly-*N*-acetyllactosamine may be correlated with the changes in cell morphology and in cell migration during neurite formation.

S16

N-Glycans of Recombinant Ancrod Expressed In Mouse Epithelial Cells Carry In Part LacdiNAc Antennae

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The thrombin-like serine protease ancrod from the venom of the Malayan pit viper *Agkistrodon rhodostoma* was expressed in mouse epithelial cells. Carbohydrate structure analyses revealed that the recombinant glycoprotein carries diantennary, triantennary and tetraantennary N-glycans with Galβ4GlcNAc-β(type-2)-antennae, part of which were further substituted by host cell-specific structural elements such as Galα3-residues or *N*-acetyllactosamine repeats. Similar to the native glycoprotein [1, 2], a substantial proportion of the oligosaccharides bore a GalNAcβ4GlcNAcβ(lacdiNAc)-branch. Again corresponding glycans were not specifically attached to a distinct *N*-glycosylation site but randomly distributed. Since other heterologous glycoproteins (e.g. recombinant tissue plasminogen activator) expressed in the same cell line do not contain such types of glycans [3], it may be concluded that expression of this structural element is due to the presence of a Pro-Lys-Lys motif which has been reported to act as a recognition determinant for the glycoprotein hormone GalNAc-transferase [4]. Possibly, this motif is also recognized by GalNAc-transferases present in mouse epithelial cells and the *A. rhodostoma* venom gland.

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S16

Molecular Cloning and Characterization of cDNAs Coding for N-Acetylglucosaminyltransferases I and II from *Xenopus laevis* Ovary

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Two different full length cDNA clones putatively coding for *N*-acetylglucosaminyltransferase I (GnT I; EC 2.4.1.101) were isolated from a lambda ZAP II cDNA library from *Xenopus laevis* ovary using degenerated PCR primers based on conserved segments from previously cloned representatives of that enzyme. The complete cDNA sequence of clone GnT I-4/3.2 revealed an open reading frame of 1332 nt coding for a transmembrane protein of 444 amino acids with a calculated molecular mass 50771. Comparison of the deduced protein sequence with that of human GnT I revealed an average similarity of 62%, with very low homology in the putative 'stem' region. In contrast, the predicted protein encoded by cDNA clone GnT I-9/7.11 does not contain a transmembrane domain. Both predicted proteins also differ in several amino acids of their putative catalytic domains. Northern blot analysis using cDNA clone 4/3.2 as a probe revealed four predominant bands of 3.0, 3.4, 4.0 and 4.8 kb with approximately equal intensities in liver, two signals with 3.0 and 3.4 kb in ovary and a single band with 4.0 kb in skin. No signal was found in lung and muscle indicating tissue-specific expression of GnT I in *X. laevis*.

Screening of the same *X. laevis* cDNA library as mentioned above using the coding region of a human genomic clone for *N*-acetylglucosaminyltransferase II (GnT II; EC 2.4.1.143) as a probe yielded ten positive clones. Clone GnT II-10/1 with a length of 2.0 kb contains the entire coding sequence for GnT II with around 65% similarity to the human enzyme within a 110 amino acid region near the C-terminus. Further DNA sequencing and characterization of selected clones are in progress.

S16

Stably Transfected BHK-21 Cells Coexpressing a Secreted Human Glycoprotein and Human α2,6-Sialyltransferase or α1,3-Fucosyltransferase; α2,6-Linked NeuAc is Attached to the 3-Branch of N-Glycans

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A stable BKH-21 cell line has been constructed by cotransfection of cells with plasmids encoding the *human* secretory glycoprotein β-trace (β-TP; [1, 2]) and *human* CMP-NeuAc: Gal(β1-4)GlcNAc-R α2,6-sialyltransferase (ST6N). Recombinant β-TP was purified from cell culture supernatants and was subjected to carbohydrate structural analysis. The enzymatically liberated oligosaccharides were found to consist of 90% of biantennary chains. About 90% of total oligosaccharides were recovered in the monosialo and disialo fractions in a ratio of 1:7. The monosialylated oligosaccharides contained NeuAc in

α2,6- or α2,3-linkage in the same ratio. Sixty percent of the disialo oligosaccharides were found to contain NeuAc in both α2,3- and α2,6-linkage to Gal(β1-4)GlcNAc-R. From ^{1}H-NMR analysis, the α2,6-linked NeuAc was shown to be attached exclusively to the Gal(β1-4)GlcNAc(β1-2)Man(α1-3)-branch of the biantennary structure. Recombinant β-TP expressed from wild-type BHK-21 cells contained biantennary oligosaccharides with α2,3-linked NeuAc only. This observed *in vivo* specificity of human ST6N supports the previously reported *in vitro* branch specificity of the bovine colostrum ST6N activity [3], demonstrating the suitability of genetically engineered mammalian host cell lines with novel glycosylation properties for the production of 'human-type glycosylated' secretory recombinant polypeptides.

A soluble form of human α1,3-fucosyltransferase VI (sFT-VI), obtained by direct PCR cloning from genomic DNA, was shown to be secreted from stably transfected BHK-21 cells. This enzyme showed activity towards sialylated N-linked oligosaccharides or glycolipids *in vitro*. Interestingly, a secreted human glycoprotein coexpressed with sFT-VI lacked any sialyl Lewis^x or Lewis^x structures.

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S16

Rat α-2,6-Sialyltransferase Gene Expression: Evidence for New mRNA Species in Fibroblasts

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The single β-galactoside α-2,6-sialyltransferase gene (α6ST(N), EC 2.4.99.1) specifies different sized mRNAs as a consequence of initiation at different tissue-specific start sites. A 4.3 kb mRNA species [1], considered at first as a liver-specific transcript, is also expected in new born rat intestine [2] whereas 4.7 kb mRNAs are found in most rat tissues and cells [3, 4]. Clones obtained from rat liver and kidney cDNA libraries indicated that these 4.7 kb transcripts possess at least two additional exons (termed E_0 and E_{-1}) in their 5′ untranslated region. To gain further insights into the regulation of the rat α-2,6-sialyltransferase gene expression, we have cloned and characterized a 4.7 kb mRNA from cultured rat fibroblasts (FR3T3 cells). In addition to the previously characterized mRNA, we have obtained an additional isoform of the 7.4 kb transcript presenting an alternative exon termed E'_{-1} in place of the previously described E_{-1} exon. To assess the tissue distribution of this new message, differential Northern blotting was performed and indicated that this new transcript is also present in other tissues. Finally, preliminary PCR data suggests that multiple transcripts of the α6ST(N) are differentially expressed according to the cell types.

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S16

Absence Of Fucosylation On Human α_1-Acid Glycoprotein In Transgenic Mice

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In this study the fucosylation of α_1-acid glycoprotein (AGP) in sera from transgenic mice carrying the cluster of the three human AGP genes [1] was studied. Crossed affino immunoelectrophoresis (CAIE) with *Aleuria aurantia* lectin (AAL) as fucose-specific affino component was used to assess fucosylation. Murine AGP was retarded in an AAL-containing gel, indicating either $\alpha1\rightarrow2$, $\alpha1\rightarrow3$, $\alpha1\rightarrow4$ or $\alpha1\rightarrow6$ fucosylation of this glycoprotein. Transgenic human AGP, however, was found to have no interaction with AAL, indicating a complete absence of fucosylation. This is in contrast to AGP in normal human sera, which is $\alpha1\rightarrow3$ fucosylated. *In vitro* fucosylation of transgenic human AGP was attempted using an $\alpha1\rightarrow3/4$ fucosyltransferase isolated from human milk. It appeared that fucose could be incorporated into this glycoprotein, as well as into normal human AGP and a non-fucosylated glycoform of AGP. This result shows that transgenic human AGP is not intrinsically resistant to fucosylation, and it also suggests that mouse liver does not express a fucosyl transferase capable of acting on human AGP.

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S16

Structural Confirmation of Lewis Antigens Immunochemically and Immunohistologically Demonstrated in Le(a-b-) Individuals

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Total non-acid glycosphingolipids were isolated from the plasma of healthy red blood cell group Le(a-b-) individuals. Glycolipids and histological tissue sections were also prepared from small intestinal epithelial cells of a Le(a-b-) nonsecretor and a secretor. Glycolipids were analysed by thin-layer chromatography, and tested for Lewis activity with antibodies reactive to the type 1 precursor (Le^c), H type 1 (Le^d), Le^a and Le^b epitopes. Tissue sections were immunohistochemically stained with the same antibodies.

By immunostaining Le^a antigens were demonstrated in most Le(a-b-) nonsecretor individuals, while Le^b could be demonstrated in all Le(a-b-) secretors.

Glycolipids from the plasma of a Le(a-b-) secretor and the small intestine of the Le(a-b-) nonsecretor were fractionated by HPLC. These glycolipid fractions were structurally characterized by mass spectrometry and proton NMR spectroscopy. In agreement with the immunostaining, small quantities of Lewis blood group glycolipids were structurally identified in the Lewis-negative individuals.

These findings confirm recent immunological evidence suggesting the production of small amounts of Lewis antigens by Lewis negative individuals.

S16

Molecular Cloning of Two Types of Rabbit β-Galactoside α1,2-Fucosyltransferase

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Two DNA clones encoding rabbit β-galactoside α1,2-fucosyltransferase (RFT-I and RFT-II) have been isolated from a rabbit genomic DNA library. The DNA sequences revealed open reading frames coding for 373 (RFT-I) and 354 (RFT-II) amino acids, respectively. The deduced amino acid sequences of RFT-I and RFT-II showed 56% identity with each other, and that of RFT-I showed 80% identity with that of human H blood type α1,2-fucosyltransferase. Northern blot analysis of embryo and adult rabbit tissues revealed that the *RFT-I* gene was expressed in adult brain, and that the *RFT-II* gene was expressed in salivary and lactating mammary glands. The activities of these enzymes were confirmed by constructing recombinant soluble fucosyltransferases followed by transfection to COS-7 cells. RFT-I expressed in COS-7 cells exhibited similar transferase activity toward type 1 (Galβ1,3GlcNAc), type 2 (Galβ1,4GlcNAc), and type 3 (Galβ1,3GalNAc) acceptors, like human H blood type α1,2-fucosyltransferase. On the other hand, RFT-II showed higher affinity for type 1 and type 3 acceptors than type 2 ones. In addition, transfection of only the *RFT-I* gene, but not that of the *RFT-II* gene, led to expression of UEA-I positive structure (type 2, H), whereas both synthesize blood group A structure. These results suggest that RFT-II is a putative secretor-type α1,2-fucosyltransferase. Other genes of rabbit α1,2-fucosyltransferase will be discussed.

S16

Enzymatic Activity of a Developmentally Regulated Member of the Sialyltransferase Family (STX) as an α2,8-Sialyltransferase toward N-linked Oligosaccharides

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We have cloned mouse STX cDNA and detected sialyltransferase activity of recombinant mouse STX, which was cloned from rat brain as a new member of the sialyltransferase family, but sialyltransferase activity of which had not been detected previously [1]. Only sialylated glycoproteins such as α_1-acid glycoprotein and fetuin served as acceptors of sialyltransfer for mouse STX. However, asialoglycoproteins and sialylated glycolipids, such as GM3, GD1a, and α2,3-sialylparagloboside, did not serve as acceptors at all. Incorporated sialic acids to sialylated glycoproteins were completely resistant against the treatments with α2,3-specific and α2,3- and α2,6-specific sialidases, respectively, but released by the treatment with α2,3-, α2,6- and α2,8-specific sialidase. In addition, N-glycanase treatment revealed that only N-linked oligosaccharides of glycoproteins were sialylated by mouse STX. However, polymerase activity for polysialic acid synthesis was not detected for this sialyltransferase. These results strongly indicate that mouse STX exhibits N-glycan α2,8-sialyltransferase activity and synthesizes Siaα2,8Sia sequences at the nonreducing terminal of N-glycan. Since the mouse *STX* gene was also highly restricted in fetal and newborn mouse brain as seen in the cases of rat and human *STX* genes, STX may be involved in the polysialylation of glycoproteins, especially of N-CAM.

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S16

Structure of Human *N*-Acetylglucosaminyltransferase-III Gene and Its Transcripts: Multiple Promoters and Splicing Patterns

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We have isolated cDNA clones for the human *N*-acetylglucosaminyltransferase-III (GnT-III) gene. Two of them, H15 and H20, contain 5′-noncoding regions that are totally different from each other except for 8 bp adjacent to the putative initiation codon. One of the genomic cosmid clones containing a GnT-III coding region, Hug3, was analysed by Southern hybridization with the use of the 5′-noncoding regions of H15 and H20 as probes, and the hybridized DNA fragments were sequenced. The 5′-noncoding regions of H15 and H20 were found to comprise two and one exons in addition to the exon containing the coding region.

Promoter activities of the genomic regions upstream of the first exons of H15 and H20, and that of the coding region were measured by luciferase assays in COS1 cells. The upstream region of the coding region was the most active, that of H15 was several times less active, and that of H20 was inactive. The absence of promoter activity in the upstream region of the first exon of H20 may be due to the difference of cell types.

The transcriptional initiation sites were determined by primer extension analysis. Both H15- and H20-specific primers gave cDNAs longer than that expected from the lengths of H15 and H20, and a primer complementary to the region around the intron/exon junction near the putative initiation codon also gave distinct signals. These results show that: 1) GnT-III mRNA consists of at least three molecular species that are different in 5′-noncoding region; and 2) the heterogeneity of the mRNA is due to multiple promoters and different splicing patterns.

S16

Influence of Flanking Amino Acids on *O*-glycosylation *In Vivo*

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We have examined the glycosylation of a secreted chimeric reporter protein by COS-7 cells. The reporter includes a known *O*-glycosylation site (PHMAQV*T*VGPGL) derived from human von Willebrand factor; *in vitro* glycosylation of this sequence is influenced by the nature of the flanking amino acids [1]. The 'wild-type' reporter was fully glycosylated, as ascertained by combinations of mild acid hydrolysis and glycosidase digestion. As expected, when the T was changed to G, the reporter did not acquire carbohydrate side chains. When the T

was converted to S, no appreciable decrease in glycosylation was observed. Flanking amino acids (−3 to +3, relative to the single threonine) were mutated to either A, P, E or R. Single alterations at positions −3, −2, +1, +2 or +3 had no effect on the glycosylation of the reporter. However, a charged amino acid at position −1 partially inhibited glycosylation (30% of the total reporter), and when combined with a charged residue at position +3, abolished glycosylation completely. Other combinations of two or more charged residues, which do not include position −1, had no effect on the glycosylation of the reporter, including a triple mutant in which E was substituted at positions −2, +1 and +2. When COS-7 cells were cultured at 23 °C to slow the passage of the reporter through the Golgi, increased glycosylation of mutant reporters (E at position −1; and E at both positions −1 and −3) was observed. Our results demonstrate that a surprisingly wide range of flanking sequences are tolerated by the *O*-glycosylation apparatus *in vivo* and suggest that both the distribution of charged residues at positions −1 and −3 and the transit time through the Golgi complex influence the level of *O*-glycosylation in cells.

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S16

Murine β1,4-Galactosyltransferase: Regulation of a Gene That Serves Both a Housekeeping and a Mammary Gland-Specific Function

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β1,4-GT is unique amongst the glycosyltransferases in that it plays a role both in glycan biosynthesis (housekeeping function in all cells) and in the synthesis of lactose (cell-specific function in the mammary gland). In mammals, the single gene for β1,4-GT specifies two mRNAs of 4.1 kb and 3.9 kb. The former is ubiquitously expressed from a housekeeping promoter, while the latter is the predominant species only in the mammary gland, and is regulated in a tissue-specific manner. We have undertaken a study to delineate the specific cis-acting elements, (and cognate trans-acting factors) governing the expression of the 3.9 kb transcript. Several protected regions adjacent to the 3.9 kb start site were identified by DNaseI footprinting analysis using nuclear extracts from various mouse tissues. Subsequent electrophoretic mobility shift assays with synthetic oligonucleotides corresponding to each of the protected regions established that four different DNA binding proteins bound to these sites. Two of these factors were found in all tissues tested, the other two were specific to the lactating mammary gland (LMG). Of the two common proteins, one was identified as Sp1, and the other recognized a GC-rich sequence in a region that was shown to be involved in the down regulation of the 3.9 kb transcript in most tissues (except LMG), and hence is a good candidate for a negative regulatory factor. The two mammary gland-restricted factors were identified as CTF/NF1 and AP-2, and both of these proteins have been shown to be important for the mammary gland-specific expression of MMTV and milk protein genes including α-lactalbumin. These data suggest an interplay of positive (both ubiquitous and cell-specific) and negative trans-acting factors in the transcriptional regulation of the 3.9 kb, β1,4-GT transcript.

S16

A New Glycosyltransferase that Acts on N-Linked Carbohydrates of the Medial Golgi

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The dominant CHO glycosylation mutant, LEC18, was selected for resistance to pea lectin [1]. Lectin binding studies showed that LEC18 cells express altered cell surface carbohydrates with markedly reduced binding to ^{125}I-PSA and a 2-3 fold increased binding to ^{125}I-DSA compared to parental cells. ^{3}H-Glc labelled LEC18 cellular glycopeptides that were desialylated and were not bound to ConA-Sepharose, exhibited an increased proportion of species that bound to a DSA-Agarose column. A fraction of the carbohydrates retarded on DSA-Agarose column that bound to a RCA_{II}-Agarose column were unique to LEC18 CHO cells. This fraction was purified from 10^{10} cells and shown by ^{1}H NMR spectroscopy and methylation linkage analysis to contain novel N-linked structures. Digestion of these LEC18 carbohydrates with β-galactosidases and β-N-acetylglucosaminidases gave core glycopeptides which, in contrast to cores from parental CHO cells, were mainly *not* bound to ConA-Sepharose. Thus ~56% of LEC18 cores contain a residue that resisted digestion and which prohibits binding of a trimannosyl core to ConA. ^{1}H NMR spectroscopy of core glycopeptides revealed new resonances consistent with a novel substitution of a GlcNAc residue in the core region. Methylation and mass spectrometric analysis of the core glycopeptides are in progress. Meanwhile a glycosyltransferase activity unique to LEC18 cells has been shown to transfer GlcNAc to a desialylated, degalactosylated, biantennary, N-linked glycopeptide—a substrate that is generated in the medial Golgi. The combined data suggest that LEC18 CHO cells express a new N-acetylglucosaminyltransferase that generates a novel N-linked core structure not previously described in the literature.

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S16

Characterization of a Rat Fucosyltransferase Gene Homologous to Human FucT-IV

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SSEA-1 (Le^{X}) positive glycosphingolipids are transiently elevated in the developing rat brain and may play an important role in the cell-cell interactions required for normal neuronal development. The final step in the biosynthesis of SSEA-1 is catalysed by specific α-1,3-fucosyltransferase(s). Although five human fucosyltransferases have been cloned and grouped into three families based on substrate specificity, chromosomal location and amino acid sequence (FucT-III, V, VI; FucT-IV; and FucT-VII), the specific enzyme involved in the synthesis of SSEA-1 glycolipids in brain has not been identified. We have previously reported isolation from rat genomic DNA of a 624 bp sequence with 89% amino acid sequence homology in the putative catalytic domain of human FucT-IV. The sequence information was used to isolate full length clones from a rat genomic DNA library (Stratagene), which were then sequenced. We now report the amino acid sequence of a full length rat clone with 78% sequence homology to the human

FucT-IV [1]. In analogy to the human gene the rat clone has two in-frame 5′ATGs [2]. Alignment of the predicted amino acid sequence between the two ATGs of the rat gene with the corresponding region of the human gene shows 65% homology. RT-PCR analysis detects the expression of this gene during early postnatal development in the rat cerebellum. Studies are now in progress to elucidate the role of this rat FucT gene in the synthesis of SSEA-1 glycolipids in developing brain.

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S16

Structure-Function Relationships of UDP-GlcNAc:α-3-D-Mannoside β-1,2-*N*-Acetylglucosaminyltransferase I

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UDP-GlcNAc:α-3-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase I (GnT I, EC 2.4.1.101) plays an essential role in the conversion of oligomannose to complex and hybrid N-glycans. Rabbit GnT I is 447 residues long and has a short 4-residue N-terminal cytoplasmic tail, a 25-residue signal-anchor hydrophobic domain, an extended stem region and a large C-terminal catalytic domain, a structure typical of all glycosyltransferases cloned to date. We now report the minimal size of catalytically active rabbit GnT I. Several GnT I constructs containing N- and C-terminal truncations were prepared using PCR and rabbit GnT I cDNA as template. The cDNA constructs were inserted into the genome of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) as a hybrid containing a mellitin signal sequence to effect secretion of the enzyme into the culture supernatant. Sf9 insect cells were infected with the recombinant baculovirus, supernatants were harvested at 4 days post-infection and assayed for GnT I activity. Removal of 106 N-terminal amino acids had no effect on GnT I activity but removal of a further 14 amino acids resulted in complete inactivation. Truncation of 7 amino acids at the C-terminus resulted in 50% loss of activity. The data indicate that the junction between the stem and catalytic domains lies between amino acid residues 106 and 120, a location which is also indicated by comparing the amino acid sequences for human, rabbit, mouse, rat, chicken and frog GnT I. The stem and catalytic domains are about 77 and 341 residues long, respectively.

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S16

UDP-GlcNAc:IV^{3}βGal-Gb$_4$Cer β-1,6-GlcNAc Transferase in Mouse Kidney; Purification, Characterization, and mRNA Expression

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UDP-GlcNAc:β-1,6-GlcNAc transferase (GNT) which transfers GlcNAc to the C-6 position of GalNAc of IV^{3}βGal-Gb$_4$Cer was purified to apparent homogeneity from mouse kidney by means of sequential column chromatographies on CM-Sepharose, UDP-hexanolamine-Sepharose, and Gg$_4$Ose-Aminocellulofine. The apparent molecular weight was 50 kDa on SDS-PAGE. The amino acid sequences of the GNT peptides generated by lysylendopeptidase digestion exhibited 80% homology with the sequence of human core2 GlcNAc transferase.

Using a 500 bp PCR product amplified by primers designed on the basis of the partial amino acid sequences of GNT as a probe, we detected GNT mRNA in not only kidney but also other tissues such as submaxillary gland and spleen. Interestingly, DBA/2 mice lacking GNT activity in the kidney did not express the mRNA in these tissues. The *GNT* gene locus was mapped on mouse chromosome 19, where the *Gsl5* gene had been mapped, by RFLP analysis of recombinant inbred strains. Since *Gsl5* gene controls the polymorphic expression of IV^{6}βGalβ1-4(Fucα1-3)GlcNAc,IV^{3}βGal-Gb$_4$Cer through the regulation of GNT activity in mouse kidney, results all together suggest that the *Gsl5* gene is the *GNT* gene itself and DBA/2 mice have a defect on the transcriptional regulation of the *GNT* gene.

S16

The Chicken Genome Contains Two Functional Non-Allelic β1,4-Galactosyltransferase Genes

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Two distinct but related groups of cDNA clones, CKβ4-GT/I and CKβ4-GT/II, have been isolated by screening a chicken hepatoma λgt10 cDNA library with the bovine β1,4-galactosyltransferase (β4-GT) cDNA clone. CKβ4-GT/I is predicted to encode a type II transmembrane glycoprotein of 41.1 kDa with one consensus site for N-linked glycosylation. A subset of clones from the CKβ4-GT/I group were identified that encode a second glycoprotein isoform distinguished by a 33 amino acid deletion in the stem region, a polypeptide segment of ~70 amino acids that links the globular catalytic domain to the single transmembrane domain. CKβ4-GT/II is predicted to encode a type II transmembrane glycoprotein of 43.2 kDa with five potential N-linked glycosylation sites. At the amino acid level the coding regions of CKβ4-GT/I and CKβ4-GT/II are 53% identical to each other and about 69% and 55% identical respectively, to bovine β4-GT. Thus the two chicken β4-GT proteins are as divergent from each other as from their mammalian homologues. Expression of each group of cDNA clones in Sf9 insect cells, using a baculovirus transfer vector, resulted in β4-GT enzymatic activity. An analysis of CKβ4-GT/I and CKβ4-GT/II genomic clones established that the intron positions within the coding region are conserved when compared to each other, and these positions are identical to the mouse and human β4-GT genes. Thus, in contrast to the mammalian genome which contains a single β4-GT gene, the chicken genome contains two functional, non-allelic β4-GT

genes that have arisen from an ancestral gene as a consequence of duplication and subsequent divergence.

S16

Differential Regulation of Glycosphingolipid Biosynthesis in Phenotypically Distinct Burkitt's Lymphoma Cell Lines

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Burkitt's lymphoma (BL) cell lines can be divided into two major groups, group I BL cell lines, which retain the original BL biopsy phenotype (expression of CD10 and CD77 antigens and lack of B cell activation markers) and group III cell lines which after several *in vitro* passages progress towards an 'LCL-like' phenotype (loss of CD10 and CD77 expression and up-regulation of the B cell activation antigens). We have shown previously that several glycolipid molecules constitute stage-specific antigens for B cells and that sequential shifts in the three major glycolipid series are observed during B cell differentiation, these changes being mostly due to sequential activations of the corresponding glycosyltransferases. Ten BL cell lines with group I or group III phenotype have now been examined for cell surface expression of five glycolipid antigens (LacCer, GM3, Gb3/CD77, Gb4 and GM2), total glycolipid content and enzymatic activities of four glycosyltransferases (GM3, Gb3, Gb4 and GM2 synthetases). We report here that group I and group III BL cells differ in their glycolipid metabolism and express either mostly globoseries or ganglioseries compounds: Gb3 is the major glycolipid of group I cells whereas GM3 and GM2 are the two major components of group III cells. These phenotypic differences are mainly due to differential activities of the glycosyltransferases: group I cells have high Gb3 synthetase activities and low or no GM3 and GM2 synthetase activities, whereas group III cells have high GM3 and GM2 synthetase activities and low Gb3 synthetase activities. Finally we also show that, unlike LCL, group III BL cells do not synthesize Gb4.

S16

Carbohydrate-Deficient Glycoprotein Syndrome Type II. An Autosomal Recessive Disease Due To Point Mutations In The Coding Region Of The *N*-Acetylglucosaminyltransferase II Gene

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Carbohydrate-Deficient Glycoprotein Syndrome (CDGS) type II is a multisystemic congenital disease with severe involvement of the nervous system associated with over 98% reduction in UDP-GlcNAc:α-6-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase II (GnT II) activities in fibroblast and mononuclear cell extracts. Eleven heterozygotes have been identified in the family of one of these patients (JV) by GnT II assays of mononuclear cell extracts. The gene for human GnT II has been cloned and is located on chromosome band 14q21. Genomic DNA was isolated from mononuclear cells and single stranded DNA from the GnT II coding region was prepared using PCR with biotin-labelled oligonucleotide primers followed by purification with magnetic beads. DNA was sequenced using fluorescent oligonucleotide primers and an automated system. Two point mutations in the catalytic domain of GnT II ($H \rightarrow R$, $S \rightarrow F$) were detected in two unrelated CDGS type II patients (JV from Belgium and MB from Iran, respectively). The father, mother and brother of JV each carry one allele with the same mutation as JV. Restriction endonuclease methods for detecting these two mutations have been developed and are being used to test DNA from 12 other members of JV's family and from 21 unrelated Belgian blood donors.

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S16

A Single Point Mutation Mislocalizes GlcNAc-TV in the Lec4A CHO Glycosylation Mutant

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The Lec4A and Lec4 CHO glycosylation mutants have been shown to lack the N-linked carbohydrate with GlcNAc(β1,6)-Man (α1,6) branches which are initiated by the GlcNAc-TV enzyme. The Lec4 mutant has no detectable transferase activity whereas that of Lec4A is equivalent to the parental CHO cells in detergent extracts. This discrepancy was explained by the demonstration that Lec4A GlcNAc-TV activity co-localizes with markers of the ER instead of the Golgi membranes. In order to understand the molecular basis of these distinct phenotypes, cDNA representing the GlcNAc-TV genes from the mutant cell lines was isolated. The Lec4 GlcNAc-TV cDNA showed a 263 bp insertion which changed the open reading frame in the 3′-end of the gene, thereby abrogating transferase activity. The Lec4A GlcNAc-TV cDNA possessed a single point mutation from T to G which resulted in a single amino acid change from Leu to Arg at position 188. The mutant and normal cDNA's were transfected into the Lec4 cell line and the transfectant phenotypes were compared. The Lec4 cDNA was unable to restore GlcNAc-TV activity to the Lec4 cell while the Lec4A cDNA converted Lec4 cells to Lec4A phenotype. Moreover, the Lec4A cDNA which had been cured of its single mutation, restored a functional GlcNAc-TV to the Lec4 cells. These and other results will be presented to demonstrate that a single amino acid change in the 747 amino acids of GlcNAc-TV can result in miscompartmentalization of the enzyme.

S16

Glycosylation of Human Monoclonal IGM is Significantly Altered by Cell Fusion

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The sugar chain structures of human monoclonal immuno-

globulin M(IgM) produced by EBV-transformed human B cell and human-human hybridoma were examined. IgM was obtained from human B cell line No. 12, and hybridoma 3-4 was established by fusing the B cell and human myeloma cell line P109. The asparagine-linked sugar chains, at each of the five glycosylation sites (Asn167, Asn330, Asn393, Asn400 and Asn560) located on the constant region of the μ chains of IgM, were isolated, pyridylaminated and subjected to the structural analysis. Based on this analysis, the structure of biantennary complex-type sugar chain, which was the predominant sugar chain at Asn167, Asn330 and Asn393, showed a different feature between the two IgM-producing cell lines examined. The biantennary sugar chain from the B cell partially (approx. 20%) lacked the β-galactose residue on the non-reducing terminal, while that from the hybridoma appeared to be fully (100%) galactosylated, indicating that the enzymatic activity of β1-4galactosyltransferase (GalTase) was insufficient for complete galactosylation in the case of the B cell.

To examine the amount of enzymatic activity of GalTase, the level of the activity in the cellular extracts from the respective cell line used was measured. The activity of the hybridoma 3-4 was several times higher than that of parental B cell No. 12, and the highest level (approx. two times higher than that of 3-4) of the activity was detected in the other parental cell, myeloma P109. These findings strongly suggested that the GalTase activity of the transformed human B cell was markedly elevated by cell-hybridization with the human myeloma showing high-GalTase expression.

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S16

Development and Application of Sensitive Solid-Phase Assays for Terminal Glycosyltransferases

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To study the enzymes responsible for catalysing the common terminal glycosylation reactions, we have developed solid-phase assays for three of these terminal glycosyltransferases termed CMPNeuAc:GalB1-3/4GlcNAc α2,3 sialyltransferase (α2,3ST), CMPNeuAc:Galβ1-4GlcNAc α2,6 sialyltransferase (α2,6ST) and UDPGal:Galβ1-4GlcNAc α1,3 galactosyltransferase (α1,3GT). Different desialylated glycoproteins were immobilized onto microtitre plates as acceptors. After enzyme reactions, we used biotinylated *Macckia amurensis* leukoagglutinin (MAL), *Sambucus nigra* agglutinin (SNA) and human anti-α-Gal IgG to detect the products for α2,3ST, α2,6ST and α1,3GT, respectively. The bound biotinylated lectins or IgG were then detected by streptavidin conjugated with either alkaline phosphatase or the bioluminescence protein aequorin. Using these sensitive assays we were able to detect these enzyme activities from different sources. We also found that these three enzymes utilized different acceptors with different efficiency. These results demonstrate that each of the three terminal glycosyltransferases tested have selective recognition of acceptor oligosaccharides in intact glycoproteins.

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S16

Molecular Cloning of Siaα2,3Galβ1,4GlcNAcα2,8-Sialyltransferase from Mouse Brain

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A cDNA encoding a new α2,8-sialyltransferase (ST8Sia III), which exhibits activity toward the Siaα2,3Galβ1,4GlcNAc sequences of N-linked oligosaccharides, was cloned from mouse brain by means of the PCR-based approach. The predicted amino acid sequence of ST8Sia III showed 27.6% and 34.4% identity with those of so far cloned mouse α2,3-sialyltransferases, i.e. GD3 synthase (ST8Sia I) and STX (ST8Sia II), respectively. Transfection of the protein A-fused *ST8Sia III* gene into COS-7 cells led to α2,8-sialyltransferase activity toward sialylated glycoproteins and α2,3-sialylated glycosphingolipids such as α2,3-sialylparagloboside and GM3. It should be noted that α2,6-paragloboside as well as asialoglycoproteins did not serve as acceptors for ST8Sia III. Surprisingly, ST8Sia III was able to synthesize GT3 from GD3. The kinetic properties of ST8Sia III revealed that it is much more specific to N-linked oligosaccharides of glycoproteins than glycolipids, even though both glycoproteins and glycolipids serve as acceptors for ST8Sia III. The expression of the *ST8Sia III* gene was tissue- and stage-specific, like the *STX* gene, but the expression pattern was clearly different from those of other α2,8-sialyltransferase genes. The *ST8Sia III* gene was expressed only in brain and testis, and the gene appeared first in 20% of embryonal brain and then decreased. Therefore, the new α-2,8-sialyltransferase is closely related in brain development.

S17. GLYCOBIOLOGY OF PROTEOGLYCANS

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Chairs: Vincent Hascall, Sakaru Suzuki

S17. 4.00pm

Cloning and Golgi Localization of Human Heparan N-Deacetylase/N-Sulfotransferases

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Heparan glucosaminyl N-deacetylase/N-sulfotransferase (NST) catalyses the initial modification reaction in the biosynthesis of mature heparan sulfate and heparin. cDNAs encoding for two human NSTs (NST-1 and NST-2) have been cloned which are

the human equivalents of the enzymes previously cloned from rat liver (NST-1) and mouse mastocytoma cells (NST-2). We hypothesized that the two NSTs reside in discrete subcompartments in the Gogli complex. A cDNA encoding a Flag-tagged human NST-1 was expressed in mouse fibroblast LTA cells and immunofluorescence detection using Flag monoclonal antibodies demonstrated that the enzyme specifically localized to the Golgi complex in a pattern similar to that of γ-adaptin, a known trans-Golgi pattern. No staining was observed at the cell surface. In addition, RNA blot analysis showed that human umbilical vein endothelial cells, IMR-90 fibroblasts, Caco-2 epithelial cells, HL-60 promyelocytic leukemia cells, and mouse mast cells all contained two NST transcripts of ~8.5 kb (NST-1) and ~3.8 kb (NST-2); the amount and ratio of transcripts varied greatly. We conclude (1) that both NST-1 and NST-2 are important for the synthesis of heparan sulfate since mRNAs for both enzymes were found in cells which do not synthesize heparin, and (2) that NST-1 is a constituent of the trans-Golgi network in LTA cells.

S17. 4.20pm

Sorting and Secretion of Heparan Sulfate Proteoglycans by Hepatocytes and Hepatoma Cells

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Using ^{35}S-sulfate as a marker, the ^{35}S-labelling of proteins in the trans-Golgi network (TGN) can be followed. The technique also allows the study of the budding and the transport of constitutive secretory vesicles, which transport the ^{35}S-labelled proteins to the plasma membrane. Heparan sulfate proteoglycans (HSPG) were almost the only ^{35}S-proteins generated by rat hepatocytes or H-35 Reuber hepatoma cells. Using pulse-chase experiments with ^{35}S-sulfate in combination with sucrose velocity gradient centrifugation and Nycodenz equilibrium density gradient centrifugation, the post-TGN vesicle population containing newly synthesized HSPG could be separated from other intracellular compartments, notably post-TGN vesicles carrying albumin, fibrinogen, and lipoproteins. The small GTP-binding protein ARF as well as a peripheral 14 kDa protein (VAPP14) and the hepatic fatty acid binding protein are associated with these vesicles. VAPP14 also exists in the cytosol and is partially associated with TGN membranes from where it is released in the presence of brefeldin A. Therefore, VAPP14 could be a coat protein. The sequence of VAPP14 shows no homologies with other known proteins. The budding of HSPG containing vesicles from the TGN was also studied in an *in vitro* system. Vesicle budding was inhibited by anti-VAPP14 antibodies. GTP-y-S as well as AlF_{4-} also inhibited the budding process, indicating the participation of trimeric G-proteins in the process.

S17. 4.40pm

Heparin Proteoglycan Activates Mast Cell Proteases and Protects them from Protease Inhibition. Is this The Physiological Role of Heparin?

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Although the pharmacological use of heparin as an effective blood anticoagulant is widespread, the physiological role of heparin is obscure. Since the presence of endogenous heparin in blood has never been demonstrated it is unlikely that heparin is a physiological regulator of haemostasis. Instead, the specific location of heparin to the secretory granules of connective tissue type mast cells and its release during mast cell activation rather points to a role in host defence. Heparin is stored in macromolecular complexes with various mast cell proteases and is released in such complexes following degranulation. In the present study we have investigated the functional consequences of the interaction between heparin proteoglycan (PG) and rat mast cell protease 1 (RMCP-1), the main chymotrypsin-like serine protease expressed by peritoneal mast cells. The interaction of heparin PG with RMCP-1 is very strong, anionic charge-dependent, and mediated by a saccharide sequence at least 14 monosaccharide units long. Heparin PG was found to activate RMCP-1 by reducing the K_m of RMCP-1 for its substrate. Further, heparin PG protected RMCP-1 from inhibition by a variety of macromolecular protease inhibitors. Also RMCP-1 present on intact mast cells, i.e. in its native complex with heparin PG showed resistance to protease inhibition. Considering the described important functional consequences of the RMCP-1/heparin interaction, and that the mast cell proteases constitute the physiological ligands to heparin PG, we believe that these findings are of significance in relation to the biological role of heparin.

S17. 4.55pm

Antithrombin-Binding Heparan Sulfate Proteoglycans are Synthesized by Ovarian Granulosa Cells

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Anticoagulant heparan sulfate proteoglycans (aHSPGs) exhibit a unique oligosaccharide sequence which specifically binds and activates the serine protease inhibitor (serpin) antithrombin III (AT). aHSPGs are synthesized by endothelial cells and endow the intimal vascular wall with anticoagulant properties. We have observed that rat ovarian granulosa cells in culture produce aHSPGs and analysed the role of aHSPGs in the extravascular compartment formed by the ovarian follicle. Affinity fractionation of ^{35}S-labelled HS glycosaminoglycan chains revealed that aHS constitutes about 6% of the total amount of granulosa cell HS. Disaccharide analysis of nitrous acid-cleaved aHS showed that they contained increased amounts of the marker disaccharides GlA → AMN-3-O-SO_3 and GlA → AMN-3,6-O-SO_3 as compared to HS which did not bind AT. Using ^{125}I-AT binding assays, we have detected aHSPGs in granulosa cell layers and culture medium, in amounts comparable to those found in endothelial cells. Stimulation of granulosa cells by the gonadotropin FSH modified the partition of aHSPGs between cell surfaces and culture medium and significant amounts of cell surface-associated aHSPGs were displaced into the medium. Such shift would correspond *in vivo* to a release of aHSPGs in follicular fluid suggesting that granulosa cells are able to regulate the localization of their aHSPGs according to their differentiation stage. At the time of ovulation serine proteases of the plasminogen activator and coagulation cascades are

activated in the ovary. These activities are tightly controlled in time and space and granulosa cell aHSPGs, as serpin cofactors, could be critically located in the ovary to modulate proteolytic activities in the growing follicle.

S17. 5.10pm

Interactions of the Neurite-promoting Protein HB-GAM with Neuron-Surface HSPG

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HB-GAM is a cell surface- and extracellular matrix-associated protein that lines developing axons *in vivo* and promotes neurite outgrowth *in vitro*. By HB-GAM affinity chromatography a heparan sulfate proteoglycan has been isolated from cultured brain neurons and from rat brain as a receptor for HB-GAM. This component has been identified as *N*-syndecan (syndecan-3). Since the neurite-promoting effect of HB-GAM is inhibited by heparin and by heparitinase, we have studied the reason for this inhibition by measuring the binding of different saccharides to HB-GAM and by evaluating their effects and the effects of extraneous *N*-syndecan on HB-GAM-induced neurite outgrowth. Studies using heparin and its modified forms, other glycosaminoglycans and oligosaccharides indicate specific binding of heparin-type glycans to HB-GAM in a manner that inhibits neurite outgrowth. A minimum of 10 monosaccharide residues is required for HB-GAM binding and inhibition of neurite outgrowth. *N*-syndecan added to the culture medium inhibits neurite outgrowth on HB-GAM coated substrates. Heparitinase treatment of *N*-syndecan abolishes the inhibitory effect. Structural studies on *N*-syndecan heparan sulfate reveal a highly *N*-sulfated structure with high proportion of contiguous IdoA-2-*O*-sulfate-GlcN-sulfate sequences. These results suggest that HB-GAM-induces neurite outgrowth via specific binding to *N*-syndecan HS-chains at the cell surface.

S17. 5.25pm

Characterization of a Glycosaminoglycan Adhesin from *Chlamydia trachomatis*

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Chlamydia trachomatis is an obligate intracellular pathogen that causes trachoma, the world's leading cause of preventable blindness, and a variety of common sexually transmitted diseases (STDs) such as urethritis, cervicitis, and pelvic inflammatory disease. *Chlamydiae* infect mammalian cells by attachment, endocytosis, and inhibition of lysosomal fusion with endosomes containing *Chlamydia*. Recent studies [1] have suggested that *Chlamydiae* attach to human host cells by a novel trimolecular mechanism involving a heparan sulfate-like glycosaminoglycan (GAG) ligand which bridges receptors on the host cell surface and chlamydial outer membrane. The GAG appears to be synthesized by *Chlamydia*, distinguishing this adhesion mechanism from other heparan sulfate-mediated microbial attachment phenomena in which pathogens bind to host cell surface heparan sulfate. The endogenous ligand can be metabolically labelled with [^{35}S]-sulfate and [^{3}H]-glucosamine, runs slightly ahead of heparan sulfate on an agarose/polyacrylamide gel stained with Toluidine Blue, and is sensitive to heparitinase. Present efforts are focused on isolation and chemical characterization of the chlamydial GAG, using strong anion-exchange HPLC, oligosaccharide mapping of enzyme digests, and electrospray ionization mass spectrometry.

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S17. 5.40pm

Tissue-dependent Alterations of Anti-Adhesive Chondroitin Sulfate-Attaching Domains of PG-M and Annexin VI, a Possible Cell Surface Receptor for the Anti-Adhesion Activity

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We previously showed that PG-M, a large chondroitin sulfate proteoglycan had an inhibitory activity for any types of cell-adhesion to substrates and suggested its general role in modulating cell-substrate interactions [1]. Several lines of evidence suggested that the activity could be due to chondroitin sulfate chains attached onto the core protein [2]. We have studied the cDNAs encoding the core proteins of chick, human and mouse PG-M. The chondroitin sulfate-attachment domains at the middle regions of the core proteins showed fairly low homology among those proteoglycans, and multiple forms of the core proteins are generated by alternative splicing of the chondroitin sulfate-attachment domains. Northern blot and RT-PCR analyses for RNA extracts from various tissues suggested a tissue-dependent alteration in the expressions of at least four different transcripts with the different chondroitin sulfate-attachment domains. Detergent extracts of tissues or cultured cells contained glycocalfin, glycoproteins of 58 kDa which were retained in both PG-M and chondroitin sulfate chain-conjugated affinity gel columns and were specifically eluted from the columns with free chondroitin sulfate or with EDTA. N-terminal amino acid sequences of fragments derived from V_8 protease-digested glycocalfin were completely identical to the reported sequences of annexin VI. Immunological comparisons using anti-annexin VI antibody also suggested the identity. Taken together, it is likely that the anti-adhesion activity of PG-M may vary from tissue to tissue by the alternative splicing, and the difference might be transduced into cells as anti-adhesion signals via cell surface annexin VI.

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S17. 6.00pm

Formation of Hyaluronan-Rich Pericellular Matrix by Arterial Smooth Muscle Cells

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The accumulation of hyaluronan (HA) and CS PG, i.e.

versican, in lesions of atherosclerosis concomittantly with smooth muscle cell (SMC) proliferation and migration suggests that these molecules play important roles in these processes. HA and PG-rich pericellular matrices can be visualized around living cells *in vitro* by exclusion of particles (fixed erythrocytes) from a halo-like region adjacent to the cells (particle exclusion assay). We have examined the synthesis and formation of pericellular coats by human aortic SMC in response to growth factors known to promote SMC proliferation (PDGF) or locomotion (TGF-β and PDGF). HA synthesis was increased in a dose dependent manner (maximum three-fold increase) in response to TGF-β1 and PDGF. PDGF AA and BB were equipotent in stimulating HA synthesis. Versican synthesis and the amount of native HA-PG aggregate was also increased in response to the growth factors. Using the particle exclusion assay, approximately 25% of cells made quiescent in DMEM + 1% human plasma derived serum (PDS) displayed detectable pericellular matrices. Addition of TGF-β1 or PDGF AB increased the proportion of cells with a matrix to 60% and 70%, respectively. The effects of TGF-β on matrix formation were additive to that of PDGF. Growth factor treatment increased not only the number of cells with a matrix but also thickness and overall size of the pericellular coats. In scratch wounded cultures of confluent cells, 85–90% of the migrating cells displayed HA-rich matrix. Cells with a motile phenotype and those in the process of rounding showed the most well developed coats, which were thickest along the sides and trailing lamellae, and less extensive at the leading edge of the cells. Time lapse imaging indicated that matrix formation occurs simultaneously with cell detachment and rounding. These data suggest that during migration and proliferation, human ASMC elaborate and organize extensive HA and PG-rich pericellular matrices which could contribute to neointimal expansion and tissue stiffness. This viscous matrix may facilitate SMC detachment, rounding, and locomotion by destabilizing interactions of the cell with fibrous matrix components.

S17 POSTERS

S17

Bladder Surface Glycosaminoglycans and Mucin Glycoproteins in Interstitial Cystitis

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Interstitial cystitis (IC) is a chronic disease of unknown etiology characterized by pelvic pain, urinary frequency and urgency, sterile urine and small bladder epithelial haemorrhages or ulcerations. Indirect evidence has implicated defects in glycoconjugates, especially glycosaminoglycans (GAGs), of the luminal surface of the bladder as a factor in the development of the disease [1]. Heparin and pentosan sulfate have been used to alleviate the symptoms in some patients. In this study we present data on the biochemical and immunochemical analysis of the glycosaminoglycans and mucin glycoproteins of human and rabbit bladders and urine. Biochemical analyses show that while the major portion of the glycoconjugates in the urothelium is sialoglycoprotein, low levels of heparan sulfate and chondroitin sulfate are also present. Immunohistochemical staining of rabbit bladder with anti-GAG (chondroitin sulfate, heparan sulfate) antibodies showed very weak staining of the luminal surface in contrast to the underlying connective tissue and blood vessels. Preliminary results with anti-epitectin (a MUC1 mucin glycoprotein) antibodies revealed weaker and patchy staining of bladder human biopsy specimens from IC patients compared to controls. The urinary GAG levels determined by an Alcian Blue precipitation assay, modified to minimize interference from Tamm Horsfall glycoprotein, were found to be lower for IC patients than healthy controls.

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S17

L- and D-Galactosyl Transferases from Snails

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Snails synthesize in their albumen glands a highly branched polysaccharide, named galactan. It is composed of D-, or D- and L-galactose (Gal). The D-Gal residues are linked $\beta 1 \rightarrow 3$ and/or $\beta 1 \rightarrow 6$ glycosidically. Only L-Gal containing galactans also exhibit $1 \rightarrow 2$ linkages.

The biosynthesis of snail galactans is not well understood. Thus far only two D-Gal transferases have been identified, one forming a linear $\beta 1 \rightarrow 6$, the other a linear $\beta 1 \rightarrow 3$ linkage. Whether L-Gal itself is attached to the core structure in this way or the L-Gal transferase requires an acceptor structure with an $\alpha 1 \rightarrow 2$ linkage, is unknown.

In the study presented here a membrane-bound D-Gal transferase was identified in the albumen glands of *Helix pomatia*, *Biomphalaria glabrata* and *Arianta arbustorum* which utilizes disaccharides as acceptors where D-Gal or D-GalNAc is in the subterminal non-reducing position and where the glycosyl residues are linked $\beta 1 \rightarrow 3$. The transfer from UDP-D-Gal is not to the terminal non-reducing position, but to the subterminal moiety, resulting in a $\beta 1 \rightarrow 6$ linkage and thus forming a branched trisaccharide.

A L-Gal transferase was also identified in the albumen glands of *Helix pomatia* and *Arianta arbustorum* transferring L-Gal from GDP-L-Gal to disaccharides, with D-Gal in the terminal non-reducing position. An $\alpha 1 \rightarrow 2$ linkage was formed irrespective of whether the terminal D-Gal was linked $\beta 1 \rightarrow 3$, 4 or 6 in the disaccharide. This finding rules out the need for L-Gal to have a special acceptor structure and proves that it is linked directly $\alpha 1 \rightarrow 2$ to the main carbohydrate portion.

S17

Neurotrophic Factors Stimulate Glycosaminoglycan Synthesis in Retinal Cells *In Vitro*

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Glycosaminoglycans (GAG) are particularly abundant in the retinal interphotoreceptor matrix (IPM), an extracellular space bordered by three cell types: pigmented epithelium (PE), Müller glia (MG) and photoreceptors (PR). We have studied

the contributions to IPM GAG content by these three types and the regulation of GAG synthesis by retinal neurotrophic factors (basic fibroblast and epidermal growth factor, bFGF and EGF respectively) using *in vitro* models. Specific GAG profiles are different for the three types: in the soluble fraction of PE, hyaluronic acid (HA) represents 70%, chondroitin and dermatan sulfate (CS and DS respectively) about 13% each; in MG, these values are 40% (HA), 16% (CS) and 40% (DS); for neurons, these values are trace for HA and DS, and 95% for CS. Heparan sulfate (HS) is always present in trace amounts. Treatment of such cultures with either bFGF or EGF induces specific changes in GAG distribution: in PE and MG, both factors lead to increases in GAG synthesis, notably with MG showing four-fold HA levels at maximal stimulation. Neuronal GAG synthesis was not affected by growth factors. These data indicate that PE and MG especially are major contributors to the retinal GAG pool, and that growth factors influence GAG synthesis in accord with their known effects on cell proliferation and migration. Hence such interactions may be relevant to retinal development, growth and pathology *in vivo*.

S17

Hyaluronate in Diabetic Skin

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Hyaluronate (HA) is an important component of the extracellular matrix. It influences numerous cellular processes. The purpose of this study was to analyse the concentrations of HA in digests of skin from 20 diabetics and 20 non-diabetic controls. HA was quantified by radioimmunoassay. There were significant decreases in HA in diabetics compared to controls ($p < 0.01$). These data suggest that HA is decreased or less available from connective tissue in diabetic skin. Glycation increases collagen rigidity and probably influences the overall packing of macromolecules, including glycosaminoglycans (GAGs) in the dermis. Glycated collagen also exhibits increased binding properties. Our results also suggest that GAGs can contribute with matrix proteins to skin elasticity in diabetes.

S17

Synthesis of Recombinant Glycosaminoglycan Chains Using the Transglycosylation Reaction of Endo-Type Glycosidases

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Methods for recombination of glycosaminoglycan (GAG) chains using the transglycosylation reaction of endo-type glycosidases were investigated in order to open a new avenue in GAG glycotechnology. Using hyaluronic acid, chondroitin, and chondroitin 4- and 6-sulfate as donors, pyridylaminated (PA) hexasaccharides prepared from the above GAGs were subjected to the transglycosylation reaction of testicular hyaluronidase, under the optimum conditions (0.15 M Tris-HCl buffer, pH 7.0, in the absence of NaCl at 37 °C for 1 h). Under these conditions, the carbohydrate chains were sequentially transferred along with disaccharide units to the nonreducing terminal of the acceptor. It was observed that the PA-oligosaccharides elongated as the reaction products increased with prolonged incubation time and the chain length of the PA-oligosaccharides reaching docosasaccharide during the 1 h incubation. Using systematic combinations of each of the above donors and acceptors under the optimum conditions for the enzyme, natural and unnatural recombinant hybrid GAG chains were synthesized. Using a combination of the above donors and GlcA-GalNAc(4-sulfate)-GlcA-Gal-Gal-Xyl-(4-methylumbelliferone) as an acceptor, which was synthesized in the cell culture system, recombinant GAG chains having proteoglycan linkage regions were synthesized. It was then possible to transfer the chains to a peptide using endo-β-xylosidase. It is anticipated that artificial proteoglycans prepared in this way will be very useful for investigating the physiological function of proteoglycans.

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2. Takagaki K *et al.* (1994) *Biochemistry* **33**: 6503.
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S17

N-Terminal Analysis of Inseparable Iduronic Acid-rich Proteoglycan Preparations from Human Skin and Post-burn Scar Tissues

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The remodelling process of the extracellular matrix of skin after burn injury involves collagen and molecules which regulate collagen fibril formation. Components in this process include the iduronic-rich proteoglycans (PGs), decorin ($PG_{(IdoA)}$-II] and biglycan [$PG_{(IdoA)}$-I]. The exact role of these macromolecules are unclear but it seems likely that decorin, at least, is involved in limiting collagen fibril diameter. Decorin and biglycan have related core proteins, containing 10 leucine-rich repeats, and differ in the number of their glycosaminoglycan chains and N-linked oligosaccharides. Scarring is characterized by disordered collagen fibrils. In order to determine whether scarring was, in part, a result of changes in the population of $PG_{(IdoA)}$s, these PGs were isolated from human post-burn normal and hypertrophic scar tissue, as well as from dermis and epidermis. Efforts to separate the two major $PG_{(IdoA)}$s type-I and II for quantitation were not successful. The different N-terminal sequences of these two iduronic acid-containing proteoglycans were utilized to estimate the relative amounts in the above isolated PG preparations from different human tissues. Normal scar, dermis and epidermis were all found to contain primarily decorin with low (<10%) levels of biglycan relative to decorin. In contrast, iduronic acid-containing proteoglycans from hypertrophic scar were found to be approximately 50% biglycan [$PG_{(IdoA)}$-I]. This may be a proximal cause of altered collagen fibrils, or may result in alterations in the sequestration of growth factors, which then result in changes in collagen that affect the appearance of the scar.

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S17

Aberrant Expressions of Extracellular Matrix Proteins in The Carbohydrate Deficient Glycoprotein Syndrome

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Carbohydrate-deficient glycoprotein syndrome (CDGS) is a congenitial disorder characterized by neurological and developmental defects. We have examined the expressions of the small proteoglycans decorin and biglycan in cultured skin fibroblasts from a patient with CDGS type 1. Northern blotting analysis identified a marked reduction in decorin mRNA and an increase in biglycan mRNA levels. The decorin protein in the culture medium was decreased. Responses to interleukin-1β (IL-1β) and transforming growth factor-β1 (TGF-β1) were apparently abnormal; decorin was only slightly up-regulated by IL-1β, while biglycan was markedly down-regulated by IL-1β and significantly up-regulated by TGF-β1. We also examined other extracellular matrix components in the fibroblast, and found high expression of fibronectin and low expression of tenascin protein as compared to normal fibroblast. The constitutional and developmental delay or the morphological changes characteristic of CDGS as well as a decreased rate of fibroblast proliferation may be associated with such derangements in the expression of extracellular matrix proteins.

S17

Size Characterization of Heparan Sulfate Degradation Products in CHO Cells

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We are using Chinese hamster ovary (CHO) cells as a model system to study heparan sulfate proteoglycan (HSPG) degradation, and the importance of the catabolic steps in the interaction of heparan sulfate (HS) with other molecules. In order to elucidate the steps in heparanase degradation of HS, it is necessary to know the sizes of the glycosaminoglycan intermediates formed in the process. We have used polyacrylamide gel electrophoresis and gel filtration chromatography to estimate the sizes of different HS species produced both *in vivo* and in *in vitro* heparanase activity assays. Commercially available heparin and chondroitin sulfate (CS) chains of known molecular weight were used as calibration standards.

Our analysis indicates the long, nascent HS chains, released from core proteins by base treatment, have a molecular weight of 130 ± 9 kDa, while the short HS chains produced *in vivo* have a molecular weight of 7.4 ± 1.1 kDa. These findings suggest that, on average, the long HS chains have 16 heparanase cleavage sites. Because intermediate-size HS chains are not observed *in vivo*, we have developed *in vitro* assays using purified HSPG substrates and partially purified CHO heparanase to follow the initial release of HS from core proteins. Our studies show heparanases cleave HS chains of 23 ± 1 kDa from the non-reducing end of the glycosaminoglycan, leaving 87 ± 4 kDa intermediates attached to core proteins. These results imply that heparanases initially produce intermediate-size HS, and these chains are subsequently cleaved to the shortest species.

Surprisingly, the CHO long HS is much larger than any HS or CS characterized to date. Because no adequate high molecular weight standards are available, this value represents only a first approximation of the long HS size. We are currently doing sedimentation equilibrium experiments to improve the molecular weight estimate of these chains.

S17

Abundance of Asialo T Antigen in Hinge O-Linked Oligosaccharide of Polymeric IGA1 and Heat Aggregated IGA1

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Gas-phase hydrazinolysis was used to analyse the glycoform of the O-glycan of fetuin and human serum IgA1. It was indicated that the quantitative analysis of the pyridylamino derivative of the released O-linked oligosaccharide from the glycoproteins was possible [1]. In this report, a method was used to compare the glycoform of O-glycan among the IgA1 subfractions. Normal human serum IgA1 could be separated into the monomeric and polymeric IgA1 on a Jacalin column. The comparative study of the carbohydrate chain between the polymeric and monomeric IgA1 showed it to be relatively rich in the polymeric form of asialo T antigen. A simultaneous analysis of the N-glycan of these fractions was also carried out. Three major identical components, two biantenna and one triantenna oligosaccharides, were obtained from both subfractions and the relative contents of these components were almost the same. On the other hand, IgA1 was artificially polymerized by heating at 62 °C for 2 h. The heat aggregated IgA1 was separated from the heat stable one using a Sephacryl S-300 column. The obtained heat stable IgA1 (approximately 30%) was not further aggregated by more heating under the same conditions. As shown in the native polymeric IgA1, the heat aggregated form also contained a much higher amount of the asialo T antigen. Thus, it was shown that the asialo type of hinge O-linked oligosaccharide might be correlated with polymerization of the IgA1 molecule. This result is very interesting since we previously reported the presence of a relatively higher content of the asialo T antigen in IgA1 from IgA nephropathy patients [2].

A part of this work was supported by NEDO.

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S17

Absence of *Helicobacter pylori* Adherence to or Interaction with Cell Surface Heparan Sulfate

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The gram negative bacterium *Helicobacter pylori* is considered

by many to be related to the development in humans of gastritis, gastric ulcer, and adenocarcinoma, with adherence to gastric epithelial cells surving as an important step in the initiation and maintenance of bacterial colonization. *H. pylori* has been shown to have some adherence to a variety of tissue culture cell lines and to organ culture explants *in vitro*, and interactions have been reported to include matrix proteins, mucoproteins, and cell surface glycolipids. Although it has also been shown that heparan sulfate binds to *H. pylori*, possible interaction of this bacterium with cell surface heparan sulfate has not been investigated. In order to determine if cell surface heparan sulfate could act as an initial receptor for *H. pylori*, we utilized HeLa cells, HS198 cells (a normal human stomach cell line), and KATO-III cells (a line derived from human gastric carcinoma) together with two ^{3}H-labelled *H. pylori* strains. Approximately 5–20% of the ^{3}H-labelled bacteria adhered to monolayers of these cells. Neither the prior addition of glycosaminoglycans (heparin, heparan sulfate, chondroitin sulfate) to bacteria or cell cultures nor pretreatment of the cells with heparinase, heparanase, or chondroitin ABC lyase had any effect in modifying subsequent adherence of bacteria. Furthermore, bacteria were not released when these same glycosaminoglycans or these same enzymes were added to cultures already containing adherent bacteria. These results suggest that heparan sulfate is not involved in the first step of *H. pylori* adherence.

S17

Uronic Acids, the Nonreducing Ends of Dermatan Sulfate

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The major dermatan sulfates of adult human skin, DS_{18} and DS_{28} [1], were digested separately by α-L-iduronidase, β-glucuronidase and β-*N*-acetylhexosaminidase. The resulting monosaccharides were separated from the rest of the polysaccharide using dialysis membranes. Quantitative analyses of the dialysates were conducted following the orcinol, carbazole and Morgan-Elson reactions to identify, respectively, IdUA, GlcUA and GalNAc.

The yields were 22.7 and 25.0 nmol L-IdUA per mg DS_{18} and DS_{28}, respectively. No D-GlcUA was obtained from DS_{18}, but DS_{28} yielded 162.3 nmol of this sugar per mg of polysaccharide. D-GalNAc was not detectable under a variety of experimental conditions.

These findings reveal the presence of L-IdUA at the nonreducing termini of both DS_{18} and DS_{28}. They also indicate that unsubstituted GlcUA occupies the nonreducing end of DS_{28}, and confirm the dermatan sulfate polydispersity. Though GalNAc was not detectable, it might be modified, and therefore unrecognized by β-*N*-acetylhexosaminidase.

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S17

Differential Distribution of two Proteolytic Fragments Derived from a Chondroitin Sulfate Proteoglycan, Neurocan

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Neurocan is a brain-unique chondroitin sulfate proteoglycan (CSPG) whose expression and proteolytic cleavage are developmentally regulated. We raised two anti-neurocan antibodies, PAb-291 and MAb-1G2, which recognize the N-terminal half (CSPG-130) and C-terminal half (CSPG-150) of neurocan, respectively [1]. Using these antibodies, we examined the spatial expression of the two proteolytic fragments in the rat brain. On postnatal day 14, both antibodies diffusely stained the cerebrum, and stained the white matter and granule cell layer of the cerebellum. However, in the mature brain, differential distribution of these two fragments was seen. In the cerebrum, CSPG-130 was localized at the cell surface of a subset of neurons and fibres, whereas CSPG-150 was diffusely distributed. In the cerebellum, CSPG-130 was localized both in the granule cell layer and in the molecular layer whereas CSPG-150 was localized mostly in the molecular layer. These results suggest that two proteoglycan fragments derived from neurocan could have different biological functions in the mature brain.

1. Matsui *et al.* (1994) *Neurochem Int* **25**: 425–31.

S17

New Synthetic Oligosaccharides as Tools to Investigate the Biology of Glycosaminoglycans

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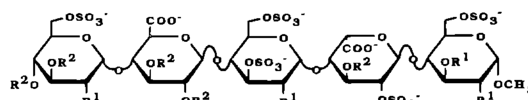
Glycosaminoglycans (GAGs) are endowed with biological properties difficult to assess because of their highly complex molecular structure. The recent finding that GAG fragments are biologically active has triggered an effort toward chemical synthesis of such fragments. However this task is still complicated owing to the presence of various substituents on the carbohydrate backbone.

To obtain more accessible structures we hypothesized that the interaction of glycosaminoglycans with their 'receptors' is essentially mediated by the charged groups present on the molecules (sulfates and carboxyls) and that the carbohydrate backbone only acts as 'scaffold'.

To test this hypothesis we have prepared three oligosaccharide analogues of the heparin binding site (1) to antithrombin (AT), a serine protease inhibitor. In compound 2 *N*-sulfates are replaced by *O*-sulfates, moreover in 3, *O*-methyl groups are also present in place of hydroxyls. The three compounds display similar biological properties (K_d for AT: 60 nM, 25 nM, and 7 nM, respectively). Thus the interaction of these nonglycosaminoglycan analogues with their target protein is maintained. Oligosaccharides of both *O*-sulfated and *O*-sulfated-*O*-methylated series constitute useful and readily accessible tools to study the biology of glycosaminoglycans.



1: $R^1 = NHSO_3^-$, $R^2 = H$; 2: $R^1 = OSO_3^-$, $R^2 = H$; 3: $R^1 = OSO_3^-$, $R^2 = CH_3$;

*This work is part of a collaboration between Sanofi Recherche and N.V. Organon, Oss, The Netherlands.

S17

Substrate Recognition by Heparanase

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Endoglycosidases (heparanases) capable of degrading heparan sulfate (HS) in the extracellular matrix are present in various mammalian cells and tissues. They have been implicated in processes such as extravasation of leucocytes, regulation of growth factor activity and metastasis. The present study was aimed at defining the substrate recognition properties of human heparanases isolated from a hepatoma cell line (SK-hep-1) and platelets.

Heparin and HS are synthesized as proteoglycans with polysaccharide (PS) chains initially consisting of D-glucuronic acid (GlcA) and *N*-acetyl-D-glucosamine (GlcNAc) units in alternating sequence. These chains become partly modified through *N*-acetyl/*N*-sulfate exchange, C5-epimerization of GlcA to L-iduronic acid units and *O*-sulfation in various positions.

Capsular PS from *E. coli* K5 has the same -(GlcAβ1,4-GlcNAcα1,4)$_n$- structure as the initial, unmodified PS precursor in heparin and HS biosynthesis. The product obtained by chemical *N*-deacetylation/*N*-sulfation and enzymatic epimerization of this PS was not cleaved by the heparanases, as indicated by gel chromatography. Incubation of the K5 PS with the sulfate donor, PAPS, in the presence of solubilized microsomal enzymes (*N*-deacetylase/*N*-sulfotransferase, GlcA C5-epimerase, *O*-sulfotransferases) from a heparin-producing mouse mastocytoma, yielded a product that served as a substrate for the heparanases. A chemically *N*- and *O*-sulfated K5 PS was degraded by the platelet enzyme. Thus, *O*-sulfate groups are essential for substrate recognition, while L-iduronic acid is not.

S17

Effect of Compound Y upon the Synthesis of an Antithrombotic Heparan Sulfate from Endothelial Cells in Culture

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Antithrombotic drugs increase the synthesis of heparan sulfate (HS) proteoglycan by endothelial cells in culture and change the sulfation pattern with a resulting increase in the trisulfated disaccharide units. Among the drugs tested, a cyclic octaphenol-sulfonic acid (compound Y), was the most powerful elicitor of the synthesis. This result led us to compare the structure of heparan sulfate synthesized by endothelial cells (C-HS) with the heparan sulfate synthesized in the presence of compound Y (Y-HS). The structure of HS has been investigated by the sequential use of degradative enzymes from *F. heparinum*. Degradation with heparinase showed different elution profiles on Sephadex G-50 with an increase of trisulfated disaccharides for Y-HS, preferentially located at the non-reducing terminal. On the other hand, the *N*-acetylated and *N*-sulfated regions at the reducing terminals of both HS are essentially the same. The increase in trisulfated disaccharides in Y-HS could be due to the activation of a 2-*O*-iduronosyl sulfotransferase or a glucosaminyl-6-*O*-sulfotransferase. Binding experiments have shown a saturation of endothelial cell receptors at a concentration of 0.16 μM for heparin and 2.7 μM for compound Y and K_s 42 nM and 1333 nM respectively. Competition experiments have shown that both compounds bind to the same receptor.

Supported by FAPESP, FINEP, CNPq and CAPES (Brazil).

S17

Enhanced Synthesis of Endothelial Cell Heparan Sulfate Proteoglycan Induced by Phorbol Ester (PMA)

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We have investigated the effect of phorbol 12-myristate-13-acetate (PMA) upon the synthesis of heparan sulfate proteoglycan (HSPG) by endothelial cells in culture. By the use of [^{35}S]-sulfate, [^{3}H]-glucosamine and [^{14}C]-amino acids we were able to demonstrate an eight-fold increase in the synthesis of the HSPG that is released to the culture medium. On the other hand chondroitin sulfate (CS) synthesis is not affected. The effect upon HSPG synthesis was totally abolished by the addition of a calcium ionophore (A23187). We have also observed that the effect of PMA upon the HSPG synthesis is more evident during the G_0–G_1 phase. When the cells were exposed to two pulses of PMA (0–1 h and 6–9 h) the amount of HSPG synthesized was almost the same as for control cells, indicating a down-regulation of the HSPG synthesis. The structure of the HSPG synthesized in the presence of PMA was also analysed and no differences were observed when compared to control. The combined data indicate that the control of the synthesis of HSPG may occur at the G_0–G_1 phase of the cell cycle and may be regulated via protein kinase C.

Supported by FAPESP, FINEP, CNPq and CAPES (Brazil).

S17

Platelet-Derived Growth Factor Stimulates Versican Expression and Galactosaminoglycan Chain Modification in Monkey Arterial Smooth Muscle Cells by Different Mechanisms

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[2]*Institute for Physiological Chemistry and Pathobiochemistry, University of Münster, Münster, Germany.*

Platelet-derived growth factor (PDGF) specifically stimulates expression of versican and increases the length and the ratio of 6-*O*-sulfate to 4-*O*-sulfate esters in galactosaminoglycan (GAG) chains of both versican and the small proteoglycans, decorin and biglycan. PDGF signal transduction is mediated by receptor tyrosine kinases (TKs) that activate protein kinase C (pKC)-dependent downstream pathways, among others. We have used inhibitors of TKs and pKC to examine if different signalling pathways are involved in the control of core protein and GAG

chain synthesis. The TK inhibitor, genistein, in a dose-dependent manner, reversibly inhibits PDGF-stimulated RNA and protein expression of versican, and the increase in ratio of 6-:4-S in the GAG chains, without affecting GAG chain elongation. Direct activation of pKC with phorbol ester (TPA) stimulates mRNA expression for versican, and GAG chain elongation, but not an increase 6-:4-S ratio in GAG chains. The pKC inhibitor, H7, but not the control analogue, H1004, inhibits both PDGF and TPA-stimulated versican expression. These observations suggest that PDGF-stimulated versican RNA expression and GAG chain elongation are pKC-dependent events. In contrast, the sulfation ratio of GAG chains is clearly pKC-independent, since TPA can neither induce an increased 6-/4-S ratio in GAG chains nor reverse the inhibition of the PDGF-stimulated increase caused by genistein.

S17

Purification, Photoaffinity Labelling and Characterization of a Bifunctional Enzyme for the Sulfation of both Chondroitin Sulfate and Keratan Sulfate

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A soluble, bifunctional sulfotransferase that could sulfate both chondroitin sulfate and keratan sulfate was purified 29 500-fold to apparent homogeneity using a sequence of affinity chromatographic steps with heparin-Sepharose, wheat germ agglutinin-Agarose and 3′,5′-ADP-Agarose. The purified enzyme exhibited a single sharp Coomassie blue-stained protein band of 75 kDa on SDS-PAGE and had a specific activity 40 times greater than the most purified chondroitin 6-sulfotransferase previously reported. Chromatography of the purified enzyme on Sephacryl demonstrated a size of 150 kDa, which indicated that the native enzyme exists as a dimer. In addition to 6-sulfation of non-sulfated GalNAc, the purified serum enzyme had the ability to sulfate GalNAc 4-S residues to give GalNAc-4,6-Di-S residues. Use of both chondroitin sulfate and keratan sulfate in the same experiment demonstrated mutual competition, establishing that the sulfation of these substrates is by the same enzyme. Photoaffinity labelling of the purified enzyme with 2-azidoadenosine 3′,5′-[5′-^{32}P]diphosphate confirmed that the 75 kDa protein is the chondroitin 6-sulfotransferase/keratan sulfotransferase. Photoaffinity labelling could be specifically inhibited by the substrates or the products of the sulfotransferase reaction.

S17

On the Total Sequence of a Heparan Sulfate from Bovine Pancreas

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A heparan sulfate isolated from bovine pancreas was subjected to the action of heparitinases I and II, heparinase, nitrous acid degradation followed by NaB^3H_4 reduction and other chemical analyses. Heparitinase I removes the *N*-acetylated and *N*-sulfated region of the heparan sulfate vicinal to the protein core. The resulting oligosaccharide from heparitinase I treatment is susceptible to the heparinase releasing GlcNS and GlcNS,6S. This indicates that these monosaccharides are at the non-reducing end and that the disaccharide IdoUA,2S-GlcNS,6S is adjacent to them. The reducing end of this oligosaccharide was formed by IdoUA-GlcNAc since $NaBH_4$ treatment followed by heparitinase II degradation releases a non-reducing unsaturated disaccharide ΔU-GlcNAc[1-OH]. Nitrous acid degradation at pH 1.5 and *N*-deacetylation with hydrazine followed by nitrous acid degradation at pH 4.0 suggest that the $(IdoUA\text{-}GlcNAc,6S\text{-}IdoUA\text{-}GlcNS)_2$ is a sequence internal and adjacent segment to the reducing end of the oligosaccharide, and the remaining block $(IdoA\text{-}GlcNS,6S)_4$ is internal and adjacent to IdoUA,2S-GlcNS,6S at the non-reducing terminal. These results are compatible with the following structure:

GlcNS(6S)-IdoA,S2-GlcNS,6S-$(IdoA\text{-}GlcNS,6S)_4$-(IdoA-GlcNAc,6S-IdoA-$GlcNS)_2$-IdoA-GlcNAc-$(GlcA\text{-}GlcNS)_5$-$(GlcA\text{-}GlcNAc)_{10}$-GlcA-Gal-Gal-Xyl-Protein core.

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S17

Potential Ligands for the Lectin-like Domain of PG-M Core Protein

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PG-M is a large chondroitin sulfate proteoglycan transiently expressed in chick limb buds at the prechondrogenic stage. The COOH terminus of the PG-M core protein contains a C-type lectin-like domain associated with EGF-like and complement regulatory protein-like domains. In this study, we have investigated the carbohydrate-binding specificity and potential ligands in the limb buds. The PG-M COOH-terminal portion was expressed in COS cells or bacteria, purified and tested for its ability to interact with different types of oligosaccharides on glycoproteins. Using a solid-phase binding assay, we showed that the COOH-terminal portion preferentially bound to complex-type oligosaccharide chains in a calcium-dependent manner. Blot assay revealed that the COOH-terminal portion bound to several glycoproteins including fibronectin in chick limb buds and that these glycoproteins contained complex-type oligosaccharide chains and/or O-linked chains. We also found that the binding properties of the PG-M COOH-terminal portion expressed in bacteria are almost the same as those in COS cells. Furthermore, affinity chromatography revealed that the COOH-terminal portion binds to heparin and heparan sulfate in a calcium-dependent manner. These results suggest that the PG-M COOH-terminal portion might contribute to the formation of the specific extracellular matrix in the limb buds at the prechondrogenic stage by the binding to some of complex-type chain- and/or O-linked chain-containing glycoproteins and heparan sulfate proteoglycans.

S17

Covalent Links Of SHAPs, Heavy Chains of Inter α-Trypsin Inhibitor-Hyaluronan Complex

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[3]*Department of Orthopedic Surgery, Nagoya University School of Medicine, Nagoya, Japan.*
[4]*Seikagaku Corp., Japan.*

We previously showed that hyaluronan (HA) synthesized by cultured fibroblasts firmly bound serum-derived 85 kDa proteins (SHAPs, serum-derived hyaluronan associated proteins). SHAPs were then identified with the heavy chains of inter α-trypsin inhibitor (ITI) [1]. In this study, the SHAP-HA complex was isolated from the pathological synovial fluids of arthritis. To elucidate the structure of the linkages, we subjected the SHAP-HA complex to limited proteolysis and hyaluronidase-digestion to obtain fragments of the linkage regions. The fragments were purified by TSK Gel HPLC and reverse phase HPLC. Peaks from reverse phase HPLC were analysed with a protein sequencer and ESI-MS. The combination with biochemical data revealed that the C-terminal Asp of each heavy chain was esterified with C6 hydroxyl group of an internal *N*-acetylglucosamine of the HA chain.

1. Huang L, Yoneda M, Kimata K (1993) *J Biol Chem* **268**: 26725–30.

S17

Sequence Analysis of Complex Chains of the Chondroitin Sulfate Type

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The chondroitin sulfate (CS) of proteoglycan is composed of a linear sequence of disaccharide units (termed A, C, D, and E*) but the content and disposition of the units varies greatly with species and anatomical sites, during development and aging, and in pathology. In order to better understand the functional significance of the structural changes, methods are being developed that enable the determination of sequences of the disaccharide units within the CS chain. The method described here for probing the structure of shark fin CS (composed of A, 26%; C, 44%; D, 21%; E, 7%) includes, as the first step, fragmentation with AC-lyase I, AC-lyase III, and C-lyase. The molar percentage of the resultant di-/tetrasaccharides were 57/40 (after AC-lyase I), 59/35 (after AC-lyase III), and 18/49 (after C-lyase). Subsequently, the tetrasaccharides were characterized and quantified by means of HPLC coupled with ABC-lyase sulfatases, and glycuronidase treatments. The molar ratio of the yields were A-D:E-D:C-E, 11:3:1, for AC-lyase I tetrasacch.; A-D:E-A:E-D, 7:2:2, for AC-lyase III tetrasacch.; C-C:C-A:D-C:D-A:C-E, 1:6:6:3:2 for C-lyase tetrasacch. Based on the average M_r 35 × 10^3, we suggest a characteristic feature of the CS chain sequence, in which the C-A-D-C sequence occurs at high frequency, the unit linked by β1,4 to D is exclusively A or E, and the D-D, D-E, E-C, and E-E sequences are entirely absent. This technique may also find application in establishing the structure of complex chains of the CS Type.

*A-unit, GlcA-GalNAc4S; C-unit, GlcA-GalNAc6S; D-unit, GlcA2S-GalNAc6S; E-unit, GlcA-GalNAc4,6-diS.

S18. SYNTHESIS AND MOLECULAR GENETIC BASIS OF DEVELOPMENTALLY-REGULATED CARBOHYDRATE EPITOPES

Chairs: Susy Glick, Jürgen Roth

S18. 4.00pm

Developmentally Regulated Expression of α2,8 Linked Polydeaminoneuraminic Acid in Rat Kidney and Brain

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We report the developmentally-regulated expression of deaminated α2,8 polysialic acid (α2,8 poly KDN) bearing glycoproteins in rat kidney and brain. Embryonic, postnatal and adult tissues were studied by immunohistochemistry and Western blot analysis with the mAb kdn8kdn recognizing this glycan with a DP ⩾ 3. In developing kidney, α2,8 poly KDN was detectable in structures derived from the two embryonic anlagen, the metanephrogenic mesemchyme and ureter bud. The metanephrogenic mesenchyme and its epithelial differentiations as well as collecting ducts were weakly positive. In adult kidney immunostaining was generally more intense and observed along the entire urinary tubulus. By Western blot analysis a single immunoreactive band of ⩾350 kDa was observed in embryonic, postnatal and adult kidney which showed an increase in intensity. Immunostaining for α2,8 poly KDN was detectable in all regions of developing rat brain. However, in adult brain immunostaining become restricted to certain defined structures. A single immunoreactive band of 150 kDa was found which became very faint in adult brain and which was distinct from polysialylated NCAM. Thus, the developmental expression profile and cellular pattern of α2,8 poly KDN and of α2,8 polysialic acid of NCAM differ greatly in kidney and brain.

S18 4.30pm

Molecular Cloning of a Novel Developmentally Regulated Sialyltransferase Specific for Sialylated Glycoproteins

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Using degenerate primers derived from the sialylmotif, a conserved motif common to all sialyltransferases cloned to date, we have cloned a novel member of the sialyltransferase gene family; STY. The cDNA sequence of STY reveals an open reading frame coding for 305 amino acids and predicts a type II domain structure consistent with the topology of other previously cloned glycosyltransferase genes. Comparison of the STY amino acid sequence with previously cloned sialyltransferases shows that it shares the lowest homology of any sialyltransferase thus far cloned. Northern analysis reveals that STY is developmentally regulated in brain with expression persisting through adulthood in kidney and spleen. Stable transfection of the full length STY clone in 293 cells produced an active sialyltransferase with marked specificity for sialylated glycoproteins with the SAα2,3Galβ1,3GalNAc sequence. Gangliosides with this sequence are poor acceptors. STY also catalyzed the addition of sialic acid to the SAα2,3Galβ1,4GlcNAc sequence found on typical N-linked oligosaccharides although with significantly less efficiency. Differential sialidase treatment and mild periodate protection experiments indicate that STY is neither an α2,6-GalNAc nor an α2,8 sialyltransferase but instead may elaborate novel types of sialyloligosaccharides. The structural elucidation of sialyloligosaccharides generated by STY is in progress.

S18. 4.45pm

Expression of α-1,3-Fucosyltransferase Genes *Fuc-TIV* and *Fuc-TVII* in Developing Myeloid Cells

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Support for the hypothesis [1, 2] that the *Fuc-TVII* gene, and not the *Fuc-TIV* gene, is important for the control of sialyl-Le^x expression on human granulocytes has come from a correlation of mRNA levels for the genes with α-1,3-fucosyltransferase activity and cell surface appearance of sialyl-Le^x in developing myeloid cells. The promyelocytic cell line HL-60 expresses Le^x strongly but sialyl-Le^x only weakly. These cells have high levels of α-1,3-fucosyltransferase activity which is directed largely towards Type 2 (Galβ1-4GlcNAc) structures that lack terminal α-2,3-sialic acid residues. mRNA from the untreated cells contains transcripts corresponding to both *Fuc-TIV* and *Fuc-TVII* genes. On DMSO-induced differentiation of the HL-60 cells along the granulocytic pathway the level of α-1,3-fucosyltransferase activity falls but the preference of the cells for non-sialylated acceptors is markedly decreased and the surface expression of sialyl-Le^x is increased. Fuc-TIV mRNA expression falls sharply on differentiation whereas Fuc-TVII mRNA expression persists. Total RNA from mature granulocytes gives clearly visible transcripts when probed with Fuc-TVII cDNA but the same amount of RNA probed with Fuc-TIV cDNA shows only very faint transcripts.

1. Sasaki K *et al.* (1994) *J Biol Chem* **269**: 14730–37.
2. Natsuka S *et al.* (1994) *J Biol Chem* **269**: 16789–94.

S18. 5.15pm

Expression of α-1,2-Mannosidase IB During Mouse Embryonic Development

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We have characterized mouse cDNA clones encoding α-mannosidase IB, a member of the class 1 eukaryotic α-1,2-mannosidase gene family [1, 2]. This enzyme trims $Man_9GlcNAc$ to $Man_5GlcNAc$ and localizes to the Golgi following transient expression in COS cells. Northern blots of adult mouse tissues and embryos show a complex pattern of tissue-specific expression of α-1,2-mannosidase IB with many transcripts of 4.2–8.7 kb. Immunolocalization with antibodies to two different unique peptides of α-mannosidase IB shows high levels of expression in specific embryonic and extra-embryonic tissue. A similar pattern of expression was observed with both antibodies. By 12.5 days, specific localization was observed in all peripheral nerves and discrete cells of sensory ganglia (dorsal root and trigeminal), myocardium and fetal liver. Intense reactions were observed in the lining cells of the labyrinthine placenta, polar giant trophoblasts, visceral yolk sac endoderm and segments of maternal endothelial cells. These observations suggest that α-mannosidase IB may play a role in placental function, and in significant events in the formation of the nervous system, heart and liver.

Supported by the Medical Research Council of Canada.

1. Herscovics A, Schneikert J, Athanassiadis A, Moremen KW (1994) *J Biol Chem* **269**: 9864–71.
2. Schneikert J, Herscovics A (1994) *Glycobiology* **4**: 445–50.

S18. 5.35pm

Developmental Roles of N-linked Carbohydrates: Generation of Mice Lacking GlcNAc-TIII

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Complex and hybrid N-linked carbohydrates synthesized by mammalian cells may possess a GlcNAc residue known as the bisecting GlcNAc. The transfer of this residue is catalysed by the enzyme *N*-acetylglucosaminyltransferase III (GlcNAc-TIII). In order to identify biological functions for N-linked carbohydrates with a bisected GlcNAc residue, we have cloned and partially characterized the mouse gene encoding GlcNAc-TIII. To investigate expression of GlcNAc-TIII in adult tissues, Northern analysis was performed and results show high levels of an ~4.7 kb RNA in brain and kidney, low levels in stomach, heart, intestine, uterus, testis, ovary and lung and undetectable levels in spleen, liver and muscle. To identify biological roles for GlcNAc-TIII the *Mgat3* gene was disrupted by homologous recombination in WW6 embryonic stem cells and germ line transmission was obtained. Mice heterozygous at the *Mgat3* locus show no apparent abnormalities, although they possess approximately half the wild-type level of both Mgat3 RNA and GlcNAc-TIII activity. We are currently mating the heterozygotes to generate homozygous mutant mice that completely lack the ability to synthesize N-linked carbohydrates with a bisected GlcNAc residue.

S18. 6.05pm

Biosynthesis of Multiply Branched Polylactosaminoglycans by β1,6-*N*-Acetylglucosaminyltransferase(s) in Hog Gastric Mucosal Microsomes

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Multiply branched polylactosaminoglycans are developmentally regulated saccharides of wide distribution. Their biosynthesis is believed to involve branching of linear $(Gal\beta1\text{-}4GlcNAc1\text{-}3)_n$ chains by β1,6-GlcNAc transferase reactions. However, multiply branched reaction products have been rarely characterized adequately. Here, we show that gastric mucosal microsomes transfer GlcNAc from UDP-GlcNAc to the pentasaccharide GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc converting it first to the branched hexasaccharide GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc, and then to the doubly branched heptasaccharide GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc. The reaction products were identified by ^{1}H-NMR spectroscopy, as well as by partial acid hydrolysis.

Supported by grants from the University of Helsinki and the Finnish Academy.

S18 POSTERS

S18

Analysis of the N-ethylmaleimide sensitive site of human α1 → 3fucosyltransferases

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Carbohydrate structures containing α1 → 3-linked fucosyl substitutions are important developmental antigens which also mediate normal leukocyte trafficking and leukocyte extravasation in inflammation. Multiple α1 → 3fucosyltransferases exist in the human genome, of which five forms have presently been cloned. These enzymes have variable substrate specificities and enzymatic properties. In an effort to define structure-function properties of human fucosyltransferases we have studied the basis for enzyme inactivation of e.g. FucT-III and -V with sulfhydryl reagents such as N-ethylmaleimide (NEM). Treatment of FucT-V with increasing concentrations of NEM resulted in effective irreversible inactivation of the enzyme. The enzyme could be protected from inactivation by inclusion of GDP-fucose or GDP but not UDP-galactose, fucose, or LacNAc in the inactivation reaction mixture. Analysis of the distribution of Cys residues in aligned sequences of cloned α1 → 3fucosyltransferase enzymes indicated one site, e.g. Cys^{143} of FucT-III and Cys^{156} of FucT-V, corresponded with the conservative Ser^{178} of FucT-IV, an enzyme insensitive to NEM inactivation. Site directed mutagenesis was performed to replace Ser^{178} of FucT-IV with a Cys. The resulting enzyme retained activity but had a three-fold higher K_m for GDP-fucose compared to the native enzyme, was NEM sensitive, and could also be protected by GDP-fucose. The results suggest that the analogous Cys residues are the NEM-sensitive, GDP-fucose protected sites in FucT-III and -V.

S18

Epithelial Cell Lineages and Glycosylation Mosaicism Revealed by Lectin Affinity Cytochemistry of Sialylated and Fucosylated Membrane Glycoconjugates in the Developing Porcine Intestine

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The temporal and spatial expression of sialylated and fucosylated intestinal membrane glycoconjugates was assessed using double fluorescent lectin cytochemistry of whole mount and semi-thin sections of intestinal villi from newborn, sucking and weaned pigs. *Sambucus nigra* agglutinin (SNA-1) labelling of α2,6-sialylated moieties on villus surfaces was high in newborn pigs, declined during sucking and was low in weaned animals. *Ulex europeus* agglutinin (UEA-1) labelling of α1,2-fucosylated moieties on villus surfaces was absent at birth, low in sucking pigs but strong in weaned animals.

The intestines in the sucking pigs revealed the interesting feature of both SNA-1 and UEA-1 labelled cells on the villus surfaces. On whole mounts, vertically orientated sheets of clonally derived SNA-1 or UEA-1-reactive cells appeared to be derived from individual crypts. These glycosylation zones were particularly conspicuous in the Peyer's patch regions of the intestine where the dome-shaped epithelium is derived from large numbers of crypts. Higher magnification analysis demonstrated cellular variations in expressed glycoconjugates, even within the apparently clonally-derived cell lineages. Cells labelled with either or both SNA-1 and UEA-1 were interspersed on the villus surfaces. This glycosylation mosaicism may reflect subtle differences of the pattern of differentiation between monoclonally derived epithelial cells on the villus surfaces. Alternatively, it indicates that the developmental transition from polyclonal to monoclonal crypts is incomplete at this early stage in porcine intestinal development.

We conclude that the developing pig intestinal epithelium has potential as a natural model system for analysis of crypt cell lineages and their modulation by dietary constituents.

S18

Multiple Branching of Blood Group i Type Poly-(*N*-acetyllactosamino)glycans by Rat Serum β1,6-N-acetylglucosaminyltransferase

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Rat serum β1,6-*N*-acetylglucosaminyltransferase catalysed the formation of the doubly branched structures GlcNAcβ1-3′(GlcNAcβ1-6′)LacNAcβ1-3′(GlcNAcβ1-6′)LacNAc (1) and LacNAcβ1 - 3′(GlcNAcβ1 - 6′)LacNAcβ1-3′(GlcNAcβ1-6′)LacNAc (2) from UDP-GlcNAc and the linear acceptors GlcNAcβ1-3′LacNAcβ1-3′LacNAc (3) and LacNAcβ1-3′LacNAcβ1-3′LacNAc (4), respectively. Only a single monobranched product, GlcNAcβ1-3′LacNAcβ1-3′(GlcNAcβ1-6′)LacNAc (5) was formed from 3, indicating that in generation

of 1 the subterminal branching occurred after the mid-chain reaction. In addition to the doubly branched saccharide 2, two monobranched isomeric glycans, LacNAcβ1-3'LacNAcβ1-3'(GlcNAcβ1-6')LacNAc (6) and LacNAcβ1-3'(GlcNAcβ1-6')LacNAcβ1-3'LacNAc (7), were formed from 4. The structures of 1–7 were determined by degradative experiments and by 1D-^{1}H-NMR; the two β1,6-linked GlcNAc residues in 1 and 2 gave distinct H-1 resonances.

Acknowledgements: This work was supported by a grant from the University of Helsinki (to O. Renkonen). We acknowledge also the cowork of Drs H. Perreault and C.E. Costello at the MIT Mass Spectrometry Resource.

S18

Developmental Changes in the Glycosylation of Glycoprotein Hormone Free Alpha Subunit During Pregnancy

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Unit on Glycobiology, Developmental Endocrinology Branch, NICHD, NIH, Bethesda, MD 20892, USA.

The α-subunit, common to the heterodimeric gonadotropin hormones LH, FSH, hCG, and TSH, is in its free form a major placental product, and has been shown to have functions that are independent of the dimeric hormones. Glycosylation of free α is essential for rendering the molecule unable to combine with the hCGβ-subunit, thus securing a population of free α molecules. Previously we have shown that glycans of free α-subunits from late pregnancy are more highly branched and contain more core-fucosylated structures than those from early pregnancy. In the present study we have analysed these changes between weeks 11 and 27 of pregnancy from five normal volunteers. Free α-subunit was isolated from 24 h urine samples by gel filtration, and analysed by lectin affinity chromatography. Binding to *Lens culinaris* increased dramatically in each of the five volunteers (mean difference: 39 ± 6%), indicating the presence of more core-fucosylated, and/or differently branched, glycan structures. In all volunteers the glycosylation change occurred at about week 15 LMP and was complete by week 17 LMP of gestation. Further studies using *Datura stramonium* lectin chromatography revealed that the type of tri-antennary branching changed profoundly in this period of pregnancy. This sudden change in glycosylation suggests developmental regulation of α6-fucosyltransferase and *N*-acetylglucosaminyltransferases IV and V. Interestingly, the activities of the same enzymes appear to be affected in malignant transformation of the trophoblast.

We conclude that glycosylation of free α-subunit changes dramatically around week 15 LMP of pregnancy. Similar changes occurred in all of the five pregnancies examined, suggesting that there is developmental regulation of the placental glycosylation machinery during normal pregnancy.

S18

Changes in the Oligosaccharide Structure of gp190, an Oncofetal Crypt Cell Antigen, upon Differentiation of CaCo-2 Cells

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gp190 is a glycoprotein expressed on the cell surface of several human colon carcinoma cells in culture, on epithelial cells of fetal colon, but not on the normal mucosa of adult colon, and thus it is referred to as an oncofetal antigen. We report here the characterization of glycans carried by gp190 synthesized by human colon adenocarcinoma CaCo-2 cells at the confluence (poorly differentiated cells) and by cells maintained in culture for 3 weeks from the confluence (well differentiated cells). By using a specific monoclonal antibody, gp190 was isolated from [^{3}H]glucosamine-labelled cells. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that [^{3}H]glucosamine-labelled gp190 from well differentiated cells migrated as a large band with a mobility lower than that of the glycoprotein from poorly differentiated cells. The glycan characterization showed that gp190 carries mainly, if not exclusively, O-linked glycans with the core-2 structure. In gp190 from well differentiated CaCo-2 cells, such a sequence appeared to be preferentially elongated by *N*-acetyllactosamine units, whereas the direct sialylation of the Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAc glycan was prevalent in the glycoprotein synthesized by poorly differentiated cells. These glycans were susceptible to digestion by neuraminidase from Newcastle disease virus, indicating that they have sialic acid in α2,3-linkage. These results demonstrated that O-glycan processing of gp190 is dependent on the state of differentiation of CaCo-2 cells.

This work was supported by AIRC and CNR, Progetto Finalizzato ACRO.

WEDNESDAY 23 AUGUST, MORNING

PLENARY LECTURES

S19. 8.00am

Nuclear and Cytoplasmic Glycosylation: It Keeps Going and Going . . .

Gerald W. Hart, Teh-Ying Chou, Man-Shiow Jiang, Kenneth D. Greis, Robert N. Cole, Frank I. Comer, Chris S. Arnold, Tatsuji Matsuoka, Doris M. Snow, Bradley K. Hayes, Lisa K. Kreppel and Betty J. Earles
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Dynamic glycosylation by O-GlcNAc is a common feature of eukaryotic nuclear and cytoplasmic proteins. Recent data substantially strengthens the notion that the saccharide plays an important role in transcriptional initiation. The *c-myc* oncogene protein is glycosylated at a site that is also a major phosphorylation site used by GSK3. Furthermore, this site on *c-myc* has by far the most abundant mutation frequency in human lymphomas. Estrogen receptors, which are ligand-inducible transcription factors, are also modified by O-GlcNAc. A major site

of glycosylation is in the PEST region of the carboxy-terminal F domain. Synthetic peptides have been used to evaluate relationships between phosphorylation and O-GlcNAc. Some appear to be substrates for both. In one example, the same site appears to be used by both modifications. Preliminary studies indicate that glycosylation of the bovine tau peptide (396-404) prevents it from being phosphorylated by MAP-kinase *in vitro*. Most interestingly, bovine brain casein kinase II alpha subunit appears to contain O-GlcNAc. Studies are underway to evaluate effects of glycosylation on kinase activity. A protein called tau, that regulates microtubule assembly in normal brain cells and forms abnormal filaments (PHF-tau) in Alzheimer's disease, has been shown to be extensively modified by O-GlcNAc. Site localization studies suggest a role for O-GlcNAc in PHF-tau formation. O-GlcNAc appears to be involved in the regulation of neurotransmitter release at nerve terminals.

Supported by NIH HD13563 and CA42486.

S20. 8.30am

Topogenesis of Sphingolipid Biosynthesis

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The Golgi apparatus of mammalian cells plays a central role both in the biosynthesis of glycoproteins and of sphingolipids. We are interested in the functional organization of the various distinct compartments that make up this organelle system. Specifically, sphingomyelin and glycosphingolipids are synthesized in individual subcompartments of the Golgi and subsequently transported to the plasma membrane. Most likely this transport occurs in vesicular carriers that bud off the Golgi and fuse with the plasma membrane, thus expressing the hydrophilic head groups of these lipids on the cell surface.

Accordingly, these head groups must be established in the lumen of the Golgi cisternae. In order to determine the sites of sphingolipid synthesis we have employed a synthetic truncated ceramide analogue with only eight carbon atoms in both the sphingosine and the fatty acid. This water soluble substrate readily permeates biological membranes and is converted into SPH, GlcCer, LacCer as well as GM_3. SPH and LacCer are synthesized in the lumen of Golgi cisternae whereas GlcCer is made at the cytosolic side of the organelle. Thus a translocator must exist in the Golgi membrane that mediates entry of GlcCer to the lumen where it can be converted to the more complex glycosphingolipids. This data will be discussed in the context of vesicular transport of these membrane lipids.

S21. 9.00am

Sponge Cell Recognition Based on Specific Glycan-Glycan Interaction

M. M. Burger[1], G. N. Misevic[1], J. Finne[2], D. Spillmann[1,2] and J. Jarchow[1]
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[2] *Department of Medical Biochemistry, University of Turku, Finland.*

The fact that cells do have inherent recognition capacities was first demonstrated in 1907 by A.V. Wilson using marine sponge cells (*Microciona prolifera*). A proteoglycan-like aggregation factor ($M_r = 2 \times 10^7$) promotes this species-specific cell-cell recognition via a Ca^{2+}-independent binding site to the cell surface and a Ca^{2+}-dependent factor-factor self association site. When aggregation factor from one species was attached to coloured beads ($0.6\ \mu\phi$) and the aggregation factor of another species to a bead of a different colour, sorting of the two beads into separate homogeneous colour clumps could be detected in preliminary experiments suggesting that these proteoglycan-like molecules suffice to explain the sorting process observed in live cells from which they were isolated. The aggregation proteoglycan consists of 950 glycans ($M_r = 6.3 \times 10^3$) which bind in the absence of Ca^{2+} to the sponge cell surface. The affinity of the single glycan was biologically irrelevant ($K_a \leqslant 10^3\ \mathrm{M}^{-1}$) but when we restored the aggregation factor size ($M_r = 1.5 \times 10^7$) by polymerizing the glycan with diepoxybutane and glutaraldehyde the polyvalent zipper-like cooperative interaction with the cell surface raised the K_a by six orders of magnitude to $1.6 \times 10^9\ \mathrm{M}^{-1}$ [1]. The other major (up to 25 chains) carbohydrate component was a large ($M_r = 200 \times 10^3$) glycan with a different sugar composition and no affinity to the cell surface but with a high affinity to itself in the presence of Ca^{2+}. The self associating strength of this glycan was able to mediate bead aggregation when they were coated with the glycan. This together with the fact that monoclonal antibodies against carbohydrate components of the glycan not only inhibited live sponge cell aggregation but also glycan coated bead aggregation suggests that recognition and aggregation of live sponge cells may be mediated via carbohydrate-carbohydrate interactions of low affinity acquiring functionality through their polyvalency [2]. Epitopes of these glycans were identified as pyruvylated and sulfated fucose containing oligosaccharides [3]. Since about 100 repetitive epitopes of one kind lie on one glycan chain, a powerful carbohydrate-carbohydrate zipper cooperativity is postulated which may mediate cellular recognition and adhesion in this system.

1. (1990) *J Biol Chem* **265**: 20577–84.
2. (1985) 3rd Intl. Sponge Conf.: 81–90, (1993) *J Biol Chem* **268**: 4922–29.
3. (1993) *J Biol Chem* **268**: 13378–87; (1995) *J Biol Chem* **270**: 5089–97.

S19. FUNCTIONAL ROLE OF O-LINKED GLYCANS WITH MUCIN-TYPE DOMAINS

Chairs: Robert Hill, Eugene Davidson

S19. 9.50am

Muc-1 Deficient Mice Develop Normally but Show Slower Growth of Mammary Gland Tumours

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MUC1 is a heavily glycosylated membrane mucin glycoprotein that is: (a) developmentally regulated; and (b) highly expressed and aberrantly glycosylated by the majority of carcinomas and in particular, by >92% of primary and metastatic breast cancers. It is hypothesized that the high level of expression of the MUC1 protein by carcinoma cells may confer an advantage upon the cell, perhaps by reducing the adhesive properties and/or by modulating the immunogenicity of the tumour cells. To test this hypothesis, we have mutated the mouse *Muc-1* locus. After analysis of greater than 400 mice, approximately 25% of the mice born from heterozygous crosses are homozygous and lack expression of Muc-1 RNA and protein. There was no obvious pathology in the tissues of the outbred (129/SvJ × C57B1/6) or inbred (129/SvJ and C57B1/6) mutant mice nor any obvious developmental abnormalities. Mice are viable at 14 months, fertile and their offspring thrive. Expression patterns of the two genes located close to *Muc-1* (*thrombospondin-3* upstream and an unnamed gene downstream) appear unaltered. Mutant mice have been bred with polyoma middle T transgenic mice to generate mammary gland tumours. Analysis of tumour growth rate shows a statistically significant decrease in tumours lacking *Muc-1* expression. Analysis of the role of the immune system in this effect is in progress. If *Muc-1* is found to confer an advantage to tumour cells, then it may be possible to down-modulate the *Muc-1* gene, possibly producing tumour cells that are more susceptible to the immune system or exhibit decreased metastatic properties.

Platform presentations (15 mins each, ≈ 5 min discussion)

10.20am–12.10pm

S19

The Role of IgA1 Hinge Region Glycans in Interactions with Cell Surface Receptors

M. Tomana and J. Mestecky
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The hinge region of IgA1 is a highly glycosylated fragment of 18 amino acids, characterized by a high content of Pro, Ser and Thr residues. The carbohydrate moiety consists of five serine-linked oligosaccharides which is either a sialylated GalNAc or mono- or disialylated Gal-β1,3-GalNAc. The hinge region glycans mediate interactions of IgA1 with cell surface receptors on several morphologically and functionally diverse cell types. We have previously shown that IgA1 binds to the asialoglycoprotein receptor [ASGP-R] on hepatocytes through hinge region glycans, resulting in the elimination of IgA1 from the circulation and its subsequent catabolic degradation, thus, they are predominantly responsible for the relatively short half-life of human serum IgA1. Our recent data suggest that hinge region glycans are also involved in the binding of IgA1 to Fcα receptors [Fcα-R] expressed on various types of cells including monocytes and granulocytes. The interactions of IgA1 with Fcα-R were studied using U937 (a monocyte-like cell line). The binding of desialylated myeloma IgA1 to U937 was significantly greater when compared to intact IgA1. Since NeuNAc is present in both O- and N-linked IgA1 glycans, this finding suggested that interactions with Fcα-R were mediated by either β1,4- or β1,3- linked terminal Gal residues. When desialylated IgA1 was subsequently degalactosylated (using galactosidase from bovine testes which predominantly cleaves β1,3-linked galactose residues) the binding of IgA1 to U937 cells decreased. The hinge region glycans also appeared to be involved in the interactions of IgA1 with a receptor expressed on mesangial cells of human kidney. Radioiodinated monomeric myeloma IgA1, modified by treatment with neuraminidase and bovine testes galactosidase, was bound more effectively to the particulate fraction of human kidney cortex and to human mesangial cells in primary culture than intact IgA1. The role of hinge region glycans in the interactions of IgA1 with receptors in the kidney was also demonstrated in *in vivo* experiments performed in mice.

Supported by NIH Grants DK49358 and DK 28537.

S19

Insect Mucin-Type Glycoprotein: Biochemical Characterization and Subcellular Localization in *Drosophila* Tissues

A. Kramerov[2], N. Arbatsky[1], E. Mikhaleva[2], O. Polesskaya[2], Y. Rozovsky[2], I. Kramerova[2] and V. Gvozdev[2]
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A secreted glycoprotein (GP) with an apparent molecular mass of 90 kDa produced by cultured embryonic cells of the fruit fly *Drosophila melanogaster* was isolated and partially characterized. GP is enriched by Ser + Thr and Pro residues that constitute up to 32% of the total number of amino acids, this feature being similar to that of highly glycosylated tandem repeat domains of vertebrate mucin glycoproteins. An abundant carbohydrate moiety (30% of molecular mass) is mainly represented by mucin-type O-linked disaccharide units Gal-(β1,3)GalNAc, occupying about one half of the total number of Ser + Thr residues and rendering the GP molecule highly resistant to protease action. A few N-glycans are also present in GP. These characteristics allow us to consider GP a member of a mucin-type glycoprotein family not yet described in invertebrates. Variations in carbohydrate structure were revealed by Western blot staining of GP with either biotinylated lectins or monoclonal antibodies with defined specificities in combination with N- and O-glycosidase treatment. A novel form of GP with molecular mass of 80 kDa was found that possessed numerous O-linked GalNAc residues still rendering it protease resistant.

Using monoclonal antibodies specific to an epitope of which the disaccharide Gal(β1,3)GalNAc is a substantial part, we immunochemically localized the fully glycosylated 90 kDa GP to a variety of *Drosophila* tissues. In many tissues GP was found to be a component of storage or secretion granules, implying its possible role in mediating the secretion process. Immunofluorescent studies revealed GP localization to ring canals involved in transport of macromolecules from trophocytes to a growing oocyte during insect oogenesis.

S19

A New Pathway for GalNAc Activation. Purification of a GalNAc-1-P Kinase and UDP-GalNAc Pyrophosphorylase from Pig Liver

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The biochemical literature indicates that GalNAc arises from and is activated via the glucosamine pathway involving amidation and acetylation of fructose 6-P to form GlcNAc-6-P and then conversion to UDP-GlcNAc which is epimerized to UDP-GalNAc. In earlier studies we purified a UDP-GlcNAc pyrophosphorylase to homogeneity from pig liver cytosol and found that this enzyme preferred UDP-GalNAc as a substrate in the backwards reaction (i.e. UDP-GalNAc + PPi → GalNAc-1-P + UTP) over UDP-GlcNAc. This enzyme also catalysed the synthesis of UDP-GalNAc from GalNAc-1-P and UTP at a faster rate (150/100) than synthesis of UDP-GlcNAc. Recently, we also purified a kinase to near homogeneity from pig kidney cytosol that catalyses phosphorylation of D-GalNAc using ATP as the phosphate donor. This enzyme has potent kinase activity towards D-GalNAc but is inactive towards Gal, GalN, GlcN or GlcNAc. Based on susceptibility of the phosphorylated product to acid hydrolysis and its resistance to $NaBH_4$ reduction, the product is GalNAc-1-P. Interestingly enough, the yeast galactokinase that forms galactose-1-P has no activity on GalNAc although it does show some activity towards galactosamine. Various properties of these two enzymes have been determined. This system may represent a salvage pathway to reutilize GalNAc arising from degradation of glycolipids and glycoproteins.

Supported by NIH HL17783.

S19

Transcriptional Control of Tracheobronchial Mucin Gene Expression

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The mucin gene is apparently up-regulated in diseases such as cystic fibrosis and asthma. To understand the mechanisms involved in transcriptional regulation of tracheobronchial mucin gene expression, we have characterized the region of the canine mucin gene (*CTM*) up-stream of the transcriptional start site and analysed the *cis*-acting elements required for mucin promotor activity. The *CTM* promoter is different from housekeeping gene promoters (as it is not rich in GC content and contains TATA- and CAAT-like sequences) and different from promoters of regulatory genes (because it contains many TATA- and CAAT-like sequences and multiple transcriptional initiation sites). Reporter gene analysis established the regions responsible for promoter activity and verified the positions of the major mucin transcriptional initiation sites. Reporter gene analysis also established that a region of the *CTM* promoter and first exon, containing all of the transcriptional initiation sites, is more active in mucin expressing cells than in mucin non-expressing cells, suggesting species specificity. The promoter region contained cAMP response element (CRE) sequences; *CTM* gene transcription was enhanced when cAMP analogs were added to transfected cells. Gel-shift assay indicated the presence of at least two DNA binding proteins which were absent in HeLa cells. One CF-specific transcription factor has also been identified.

Supported by the Cystic Fibrosis Foundation and NIH.

S19

Physical and Biochemical Changes in Cervical Mucins through the Ovulatory Cycle

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There are changes in the rheological properties of cervical mucus through the ovulatory cycle. At midcycle there is a pronounced drop in mucus viscoelasticity associated with an increase in the hydration of the gel. These changes are correlated directly with sperm penetration, however it is not clear that these changes in the mucus are in any way related to changes in the cervical mucins. We and others have previously demonstrated the presence of a major family of mucins that are deemed responsible for the mechanical properties of the gel. In this study we have isolated these glycoproteins from: pregnancy mucus both at term and taken pre-term before abortion; mucus harvested at midcycle and mucus collected daily through the ovulatory cycle. The mucins in each case appear to be predominantly the MUC 5 gene product as identified by using an antiserum raised against a synthetic peptide. Comparisons of midcycle with pregnancy mucins indicate an increase in the polydispersity of the former. At midcycle the mucins have a broader M_r distribution (assessed by rate zonal centrifugation) and a higher average mass and size (assayed by dynamic and absolute light scattering measurements).

Glycopeptides prepared from midcycle mucins by reduction of disulphide bonds followed by trypsin digestion were distinctly less acidic on anion-exchange chromatography as compared with glycopeptides prepared from cervical pregnancy mucins. Similar analyses were performed on the mucins purified from cervical mucus collected daily through the ovulatory cycle. The data indicate a cyclical change in mucin acidity, the molecules being substantially less charged at days 12–14 of the cycle. It is not yet clear whether different mucin glycoforms are secreted at midcycle or if the molecules are modified extracellularly.

S19

Individual Variation in Human Conjunctival Mucins

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Isolated and partially purified mucin from mixed collections and individual samples of cadaver conjunctiva have been compared by agarose gel electrophoresis and Western blotting. Purification was achieved by CsCl density gradient centrifugation and gel filtration. Three buoyant density ranges were studied separately: $d < 1.35$, 1.35–1.45, 1.45–1.6 g ml^{-1}. Mucin containing fractions were identified by cross-reaction with lectins and anti-mucin peptide core antibody (aM1). They were found in both the excluded and the larger included fractions of Sepharose CL2B columns. The strongest cross reactivity was observed in the classical mucin density range: 1.35–1.45 g ml^{-1}. Western blots of agarose gel electrophoresis of native and reduced-alkylated mucins showed no difference in mobility between individuals and combined samples. Native samples displayed a similar degree of polydispersity. Reduced-alkylated fractions appeared to contain fractions with migratory characteristics close to the parent sample, and a much faster component reacting with WGA. Cross reaction with aM1 was markedly diminished following reduction-alkylation, and appeared as two distinct bands. Cross reaction with antibodies against Tn and sialyl-Tn antigens also appeared weaker after reduction and alkylation. This weakening can be attributed to loss of material during the procedure. Individual variation in human ocular mucins probably occurs at the level of oligosaccharide chains without affecting the overall charge-mass partition of the molecule.

S19 POSTERS

S19

Biosynthetic Studies on the Human MUC2 and MUC5 Mucins

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The biosynthesis of the human intestinal mucin MUC2 was studied in the colon adenocarcinoma cell line LS 174T. Using an antiserum against the tandem repeat of the mucin in immunoprecipitation, we estimated the primary translation product of the mucin to approximately 700 kDa as determined by SDS-PAGE [1]. This monomeric form of the apoprotein forms a dimer after 30 min chase, via disulphide bonds. Dimerization starts before *O*-glycosylation as indicated by data from both brefeldin A treated cells and cells immunoprecipitated with the *Helix pomatia* lectin. Immunoprecipitation studies on tunicamycin treated cells showed that the dimerization of MUC2 is less efficient if *N*-glycosylation is inhibited.

Our results also show that further oligomerization of the dimer takes place after *O*-glycosylation. These results suggest that the biosynthetic pathway of MUC2 may be similar to that of the von Willebrand factor with which MUC2 shares sequence homologies in its C- and N-terminal ends.

Comparisons of the biosyntheses of MUC2 and another human intestinal mucin, MUC5, indicate that the two mucins have different synthetic pathways. MUC2 appears to form only a dimer before *O*-glycosylation, whereas the MUC5 mucin appears to form trimers and maybe even tetramers before GalNAc-glycosylation. MUC2 and MUC5 are both synthesized by the same human colon carcinoma cell lines, but no kind of heterodimerization between the two mucins has been detected.

1. Asker N, Baeckström D, Axelsson MAB, Carlstedt I, Hansson GC (1995) *Biochem J* (in press).

S19

Structure and Properties of the MUC5 and MUC2 Mucins from Human Gastrointestinal Tract

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[2]*Department of Surgery, Lund University Hospital, Sweden.*

The large secreted mucins form a biofilm – mucus – that protects the epithelial surface. In the stomach, mucins are produced by the surface epithelium and by submucosal glands. The presence of secretory cells with different histochemical properties suggests the presence of mucin populations with different glycosylation.

Gastric mucins were isolated from mucosal scrapings of human stomachs with density-gradient centrifugation in CsCl/guanidinium chloride. A major population was identified as the MUC5 mucin by using polyclonal antibodies raised to a synthetic peptide. The macromolecules are large and composed of subunits as shown previously for other mucins. In antrum, one of two populations reacted strongly with antibodies against the Le^b structure showing the presence of mucins with different glycosylation. Furthermore, populations with 'high' and 'low' reactivity with Alcian Blue were identified in keeping with the histochemical appearance of the tissue.

The major part of rat small intestinal mucins occurs as a glycoprotein complex that is 'insoluble' in e.g. guanidinium chloride [1]. Mucosal scrapings from human colon were extracted with guanidinium chloride and the extraction residue was subjected to reduction in order to solubilize the insoluble glycoprotein complex, if present. Isopycnic density-gradient centrifugation showed that almost all mucins were present in the extraction residue. Polyclonal antibodies raised to four different synthetic peptides from the MUC2 sequence reacted with the mucin subunits from this complex. It is concluded that MUC2 is the dominating mucin in human colon and that this mucin occurs as an insoluble glycoprotein complex as in the rat analogue.

1. Carlstedt *et al.* (1993) *J Biol Chem* **268**: 18771–81.

S19

Towards an Understanding of Mucin Glycosylation in Human Colon Cancer Cells: Effect of Benzyl-*N*-acetyl-*α*-galactosaminide

Philippe Delannoy[1], Isabelle Kim[2], André Verbert[1], Pierre Degand[2] and Guillemette Huet[2]
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We have analysed mucin expression in the secreting HT-29-

MTX cell line (derived from the HT-29 human colon carcinoma cells through a selective pressure with methotrexate [1]) in relation to the presence of benzyl-*N*-acetyl-α-galactosaminide (GalNAc-α-Bz) which is a potential competitive inhibitor of the β-1,3-galactosyltransferase that synthesizes the T-antigen.

The main observation was a dramatic decrease in the sialic acid content of mucins which is 13-fold lower after 24 h of exposure to 5 mM GalNAc-α-Bz. This was consistent with the increased reactivity of these mucins to Peanut lectin, testifying to the higher amount of T antigen. The second observation was a decrease in the secretion of the mucins by GalNAc-α-Bz treated cells.

We show that this decrease in mucin sialylation was achieved through *in situ* β-galactosylation of Gal-NAc-α-Bz, leading to the formation of Galβ1-3GalNAc-α-Bz which acts as a competitive substrate of Galβ1-3GalNAc α-2,3-sialyltransferase, in agreement with the observed accumulation of NeuAcα2-3Galβ1-3GalNAc-α-Bz, together with other compounds, into treated cells. We are examining whether the decrease in secretion is associated with the level of sialylation of the mucins in HT-29-MTX cells.

1. Lesuffleur *et al.* (1991) *Cancer Res* **50**: 6334–43.

S19

Glycosylated Regions of Pig Colonic Mucin

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The mucin genes sequenced so far have different structures and by implication different functions. The *MUC2* gene product comprises two highly glycosylated regions separated by a sparsely glycosylated region. The *MUC5AC* gene product is comprised of a series of repeating heavily and sparsely glycosylated regions. To date two pig mucin genes have been partially cloned, pig submaxillary mucin (PSM) and pig gastric mucin (PGM). The tandem repeat region of these two genes in contrast to those so far reported for human mucins contain a greater proportion of serine than threonine.

The molecular weight of the pig colonic mucin reduced subunit was determined to be $\sim 2 \times 10^6$ and that of the digested subunit $\sim 6 \times 10^5$. This would suggest that the pig colonic mucin subunit is comprised of two to three highly glycosylated regions separated by sparsely glycosylated regions. This structural arrangement has also been suggested for other mucins from physiochemical analysis and is similar to the structure assumed for *MUC5AC* gene product.

Papain digested pig colonic mucin comprised in total 42% Ser, Thr and Pro, comparable to that reported for PSM tandem repeat region (45%). The proportion of proline in the digested mucin 9% was comparable to that found in the tandem repeat regions of PSM and PGM (both 6%). Unlike PSM and PGM tandem repeat regions which contain a greater proportion of serine than threonine, there were approximately equal proportions of threonine and serine in digested pig colonic mucin.

Papain digested pig colonic mucin was deglycosylated with TFMS. Deglycosylated peptides were separated on the basis of hydrophobicity by HPLC using a phenyl 5PW reverse phase column and a linear gradient of 5–80% acetonitrile in 0.05% TFA. In preliminary studies three mucin peptide fractions with different amino acid composition were separated.

S19

Further Characterization of Human Middle Ear Mucins

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Mucins determine the viscosity of the fluid which accumulates in the middle ear cleft of children with Otitis Media with effusion. Previous studies have shown that the rheological properties of effusions vary with mucin concentration and with differential secretion of different mucin species. Middle ear mucosa is a modified respiratory epithelium and at least four mucin genes so far described are expressed in the airways (MUC 2, 4, 5AC and 5B). The fragmentation pattern on reduction and proteolysis of the well characterized mucin from thick effusions is most compatible with the predicted structure of MUC 5AC.

In the current study mucin was purified from three pools of effusions: (1) thick and (2) thin effusions, from anatomically normal children, which have significantly different viscosities; and (3) effusions from children with cleft palate. Purified mucin was proteolytically digested to produce the heavily glycosylated domains equivalent to the tandem repeat regions of mucin genes.

Amino acid analyses of digested mucin from the three pools were different. Serine, threonine and proline contributed 44%, 29% and 36% of the protein cores of mucins, from groups (1) (2) and (3) respectively, in the ratios (2.4:2.9:1) (5:1:1) and (3.5:1:1.5) suggesting the expression of different genes. The serine to threonine ratio in thick effusions is similar to that found in MUC 4, however, the high proportion of serine in mucin from groups (2) and (3) is unusual.

Further differences in the protein cores were observed after deglycosylation. Polymeric mucins from groups (1) and (2) were deglycosylated with TFMS. SDS PAGE under reducing conditions gave a diffuse band of M_r 70–80 kDa for apomucin from (1) while that from (2) gave a larger M_r band at the origin of and spreading into an 8–25% gel suggesting the protein core of thin mucin is larger.

S19

Effect of Endo-GalNAc-ase-S from the Culture Medium of *Streptomyces* sp. OH-11242 on Sialoglycoprotein

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The endo-α-*N*-acetylgalactosaminidase from a culture medium of *Streptomyces* sp. OH-11242 (endo-GalNAc-ase-S) has been partially purified, and its specific activity, molecular mass, and some characteristics have been reported. In the present study, it was determined whether endo-GalNAc-ase-S liberated sialyl

oligosaccharides from fetuin and epitectin derived from human laryngeal carcinoma cells. The crude endo-GalNAc-ase-S precipitated with 80% (w/v) ammonium sulfate from the concentrated culture medium was further purified by gel chromatofocusing and subsequent *N*-(p-aminophenyl)oxamic acid-agarose chromatography. The partially purified enzyme was incubated with fetuin in the presence of 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid. The reaction mixture was fractionated using a Bio-Gel P-4 column, and each fraction was assayed using the thiobarbituric acid method. A portion of the sialyl oligosaccharide fractions was pyridylaminated, and then analysed by HPLC. Endo-GalNAc-ase-S hydrolysed the O-glycosidic linkage between GalNAc and Ser (Thr) in fetuin liberating three kinds of the oligosaccharides, NeuAcα2-3Galβ1-3GalNAc, NeuAcα2-3Galβ1-3[NeuAcα2-6]GalNAc, NeuAcα2 - 3Galβ1-3[NeuAcα2-3Galβ1-4GlcNAcβ1-6]GalNAc. The molar ratio of the three oligosaccharides was different from the molar ratio of O-linked oligosaccharides in fetuin which has already been determined. When the epitectin was incubated with endo-GalNAc-ase-S, sialyl oligosaccharides were obtained.

S19

Isolation and Physical Characterization of Salivary Mucin-Glycoprotein MG2 and its Interaction with Oral Bacteria

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Several studies have implicated salivary mucins in important roles modulating bacterial colonisation of oral surfaces. Mucins are secreted by the (sero) mucous salivary glands. There are two chemically distinct mucin populations in whole human saliva designated MG1 and MG2. The latter has recently been cloned and sequenced and is now designated MUC 7. Studies have shown that glycans present on MG2 can act as ligands for lectins present on the bacterial surface. In order to further investigate these interactions MG2 was purified to homogeneity. This was accomplished by a rapid two stage protocol employing gel filtration on Sepharose CL-4B followed by HPLC ion exchange chromatography on Mono Q.

The molecules have been characterized by gel chromatography, analytical ultracentrifugation, light scattering and electron microscopy. Gel chromatography indicates that the purified molecules have a marked tendency to self-associate under physiological conditions. Electron microscopy suggests that MG2 assembles by an end to end association into long filamentous aggregates. Experiments performed in 4M guanidinium chloride visualise the molecules as rod-like with an average length of 62 nm.

Interactions of *Streptococcus gordonii, S. anginosus, S. crista, and S. mutans* with MG2 are being studied with direct binding assays. MG2 has been covalently linked to thiol Sepharose for affinity isolation of bacterial surface adhesins. Data concerning these studies will be reported.

S19

Single-site Glycosylation in Mucin Domains

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Mucins are glycoproteins which can comprise up to 50% sugars which are located in repeating domains containing long sequences of glycosylated amino acids. The function which these O-linked sugars have in mucins has been difficult to define in terms of both their physical properties and their functional specificity since the analysis of these sugars has been restricted to methods which totally remove the sugars from their sites of attachment to the protein. Only the average composition of the oligosaccharides on the mucins can be determined by this approach.

Until now, the only method available to characterize the oligosaccharides attached to a particular amino acid in a glycoprotein required the isolation of a glycopeptide which contained a single site of glycosylation. The oligosaccharide(s) at the single site could then be analysed. This is not possible for mucins in which the glycopeptides are composed of amino acid sequences which contain many clustered sites of glycosylation.

Solid-phase Edman degradation using a Beckman LF 3600 protein sequenator can sequence through these heavily glycosylated domains yielding PTH-glycoamino acids which can be quantified and then analysed for their sugar content by HPAEC-PAD and by ES-MS.

Using these techniques we were able to identify the sugars attached to the individual amino acids in the mucin-like domains of glycophorin A and a recombinant cell surface protein of *Dictyostelium*(PsA) and other mucin domains.

The characterization of the oligosaccharides on individual amino acids in these heavily glycosylated domains of mucins will greatly assist in assigning the functional importance of the sugars in these proteins.

S19

A Major Airway Mucin Gene, *MUC8*: Chromosomal Localization and Cloning of Complementary DNA

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A partial cDNA encoding a major airway mucin glycoprotein with novel tandem repetitive sequence has recently been cloned [1]. We describe here the sequence of the carboxy terminus of complementary DNA derived by 3′-RACE. Compiled together, this partial cDNA extends to a 1420 bp sequence with a single open reading frame of 944 bp and a 3′-UTR of 458 bp. A 5′-end 325 bp fragment subcloned into the protein fusion and expression vector pET28b(+) was used to generate fusion protein under the control of T7 promoter. Antiserum raised against purified fusion protein reacted with deglycosylated airway mucin, proving that the cloned cDNA encodes an apomucin. Additional confirmation was achieved by showing that polyclonal antibodies to a synthetic peptide corresponding to the MUC8 repeat sequence TSCPRPLQEGTPGS also reacted with purified deglycosylated mucin. Immunohistochemical studies

using the above antibodies localized the MUC8 protein to both goblet cells and submucosal glands in tracheal epithelium. Further, the gene from which this cDNA is derived was mapped to chromosome 12 in the region 12q24.3 using human-mouse somatic cell hybrids and *FISH* technique, respectively. Since this mucin gene maps to chromosome 12, different from the other seven human mucin genes known so far, we propose to name this *MUC8*.

Supported, in part, by NIH grant HL34012.

1. Shankar V, Gilmore MS, Elkins RC, Sachdev GP (1994) *Biochem J* **300**: 295–98.

S19

Identification and Characterization of the Mucin MUC 5 in Respiratory Secretions: Differences Between 'Normal' and Disease Secretions

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We have isolated the mucins from an asthmatic mucus by isopycnic density gradient centrifugation. The mucin fraction ($\rho = 1.35–1.50\ \mathrm{g\,ml^{-1}}$) was reduced and alkylated to yield reduced mucin subunits that were fractionated by anion-exchange chromatography on Mono Q and 1% agarose gel electrophoresis. At least three different populations of mucin subunit were identified using lectins and a range of antibody probes. These populations were purified to homogeneity (as assessed by agarose electrophoresis) by Mono Q chromatography. A polyclonal antiserum raised against a synthetic peptide of MUC 5 identified one of these components which accounted for approximately 15% by weight of the total mucin preparation. The major mucin in this secretion (77% by weight) was not recognised by the antiserum and has a different amino acid composition to the MUC 5-positive component. The MUC 5 antiserum has also been used for immunoprecipitation and epitope-localization using immunoelectron microscopy. Using isopycnic density gradient centrifugation we have isolated respiratory mucins ($\rho = 1.35–1.50\ \mathrm{g\,ml^{-1}}$) from 20 'normal' individuals as well as those from individuals with different hypersecretory conditions. We have observed marked changes in mucin glycosylation (compared with normal) associated with infection and inflammation as well with hypersecretion associated conditions such as asthma and chronic bronchitis. Our observations suggest the presence of at least two major gene products occurring in different but distinct glycosylated forms. MUC 5 accounts for approximately 60% of the mucins in 'normal' secretions whereas in some chronic hypersecretors it is only a minor component (less than 10%). However whenever we find MUC 5 in respiratory secretions it appears as the same biochemical entity (as assessed by Mono Q chromatography and agarose electrophoresis) regardless of the individual or their state of health. This suggests that this particular mucin gene may have a specific pattern of glycosylation associated with it in the lung.

S20. ORGANIZATION AND TRAFFICKING OF GLYCOSPHINGOLIPIDS IN MEMBRANES

Chairs: William Young, Richard Pagano

S20. 9.50am

How Does GPI-Anchor Addition Regulate Protein Exit from the Yeast ER?

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Yeasts, like all higher eukaryotes, modify a subset of their polypeptides by the addition of glycosyl phosphatidylinositol (GPI) anchors. These anchors may play a role in intracellular protein traffic, possibly by associating with each other and with cellular glycolipids [1]. One GPI-anchored protein in *Saccharomyces cerevisiae* is an abundant 125 kDa surface protein, Gas1p [2]. In the absence of GPI addition, Gas1p fails to acquire Golgi modifications, apparently not progressing further than the endoplasmic reticulum (ER) [3]. We are interested in the relationship between GPI addition and the mechanism of protein exit from the ER.

Yeast cells starved for inositol demonstrate defects in maturation of both Gas1p and other protein markers (e.g. carboxypeptidase Y, alkaline phosphatase). The defect of Gas1p maturation, however, is more rapid in onset than that of the other proteins, suggesting that it is caused by a more immediate process, distinct from any global secretory alteration induced by starvation conditions. The phenotype is readily reversible *in vivo* by inositol addition. Inositol starvation also induces a rapid decrease in GPI biosynthesis as judged by examination of biosynthetic intermediates made *in vitro*.

Immature Gas1p may be detected as cargo in ER-derived transport vesicles released from yeast membranes *in vitro*. We are currently examining how such transport is altered under conditions of inositol starvation. This *in vitro* approach will allow investigation of the interactions between GPI-anchored proteins and components of the yeast secretory apparatus.

1. Simons, Wandinger-Ness (1990) *Cell* **62**: 207.
2. Fankhauser, Conzelmann (1991) *Eur J Biochem* **195**: 439.
3. Nuoffer *et al.* (1993) *J Biol Chem* **268**: 10558.

S20. 10.10am

Glycosphingolipid Synthesis Using Cloned Glycosyltransferases

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Glycosphingolipid (GSL) synthesis takes place in the Golgi secretory pathway of mammalian cells. Data regarding the kinetic and spatial relationships between individual steps of

GSL glycosylation are limited. We have begun studies using cloned glycosyltransferases specific for GSL synthesis to address these issues. We stably transfected Chinese hamster ovary (CHO) cells with epitope tagged glycosyltransferase genes and with constructs designed to direct each enzyme to various points in the secretory pathway. Results with cloned β1,4 *N*-acetylgalactosaminyltransferase (GM2 synthase) suggest that this enzyme became disulfide bonded, perhaps as a homodimer, early in the secretory pathway and was released to the culture medium later in the pathway. Synthesis of product GSL *in vivo* occurred if GM2 synthase was located in the Golgi but not if the enzyme remained in the endoplasmic reticulum.

S20. 10.30am

Glycosphingolipids, GPI-Anchored Proteins, and Acylated Proteins Cluster in Membranes through Acyl Chain Interactions

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Glycosphingolipid-rich membrane complexes that contained proteins anchored by glycosylphosphatidylinositol (GPI) were isolated from mammalian cell membranes based on their insolubility in non-ionic detergents. Model liposomes with a similar lipid composition called SCRL (sphingolipid/cholesterol-rich liposomes; 2:2:1:1 phosphatidylcholine:cholesterol:glucosylceramide:sphingomyelin, mole ratio) were also not fully solubilized by detergents. The propensity of glycosphingolipids, sphingomyelin, and biological phospholipids to resist extraction from these and other liposomes correlated with their acyl chain melting temperatures. Tracer amounts of dipalmitoylphosphatidylcholine (DPPC) ($Tm = 41°$) were not solubilized well from SCRL, while dioleoyl phosphatidylcholine ($Tm = -20°$) was extracted. The 'fluidity' of the detergent-resistant membranes, as measured by diphenylhexatriene fluorescence polarization, was similar to that of liquid-ordered membranes. Liquid-ordered phase liposomes (DPPC: cholesterol 2:1) were also detergent resistant. A purified GPI-anchored protein was not extracted from SCRL by detergent. Most GPI-anchored proteins contain two saturated acyl chains, which should associate with liquid-ordered domains. We propose that glycosphingolipids and GPI-anchored proteins associate in domains in SCRL and possibly in cell membranes, and that these domains may be in a cholesterol-rich liquid-ordered phase. The glycosphingo-lipid-rich domains appear to be present in non-clathrin-coated pits called caveolae in cells that contain these structures. Several members of both the Src-family non-receptor tyrosine kinase family and the heterotrimeric G protein family are modified by two saturated acyl chains, myristate and palmitate, in close proximity, and are also present in detergent-resistant membranes. These acyl chains may target proteins to glycosphingolipid-rich domains in cell membranes.

S20. 10.55am

Postendocytotic Glycosylation and Retrograde Transport of Glucosylceramide and Lactosylceramide Analogs that are Resistant to Degradation

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We have synthesized radioactive and fluorescent analogues of glucosyl- and lactosylceramide that contain sulfur as glycosidic and interglycosidic link, respectively, and that are thus resistant to enzymatic deglycosylation. The structures of these compounds as well as those of their synthetic intermediates were confirmed by FABMS, NMR and/or elemental analysis. Their resistance to enzymatic hydrolysis by GM1-β-galactosidase and glucocerebrosidase was established under conditions that readily led to hydrolysis of their O-glycosidic counterparts. The labelled analogues were complexed to defatted BSA and used to study their uptake by and metabolism in cultured cells (human fibroblasts, rat neuroblastoma B 104, human neuroblastoma SHSy5y) and murine primary cerebellar neurons. These glycolipid analogues were easily taken up by these cells and were considerably glycosylated to yield a cell-type specific pattern of glycolipid analogues. In fibroblasts labelled globosides and gangliosides GM3, GD3 and GD1a were predominantly formed. In B 104 cells almost exclusively GM3 was produced whereas in human neuroblastoma cells GM2 was the main product formed besides GM3, GD3 and GD1a. In murine cerebellar cells gangliosides GM1, GD1b and GT1b were also formed. These results clearly indicated for the first time that glycolipids incorporated into the plasma membrane of various cells can be transformed to Golgi compartments involved in the early glycosylation steps of glycolipids. This indication is confirmed by the fact that monensin inhibited the formation of GM2, GM1, GD1a, GD1b and GT1b but not of GM3 and GD3.

S20. 11.15am

Globotriaosyl Ceramide-Mediated Intracellular Targeting of Verotoxin. Retrograde Transport to the Nucleus and Beyond

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The B subunit of verotoxin (VT) mediates the attachment to cell surface globotriaosyl ceramide (Gb_3) to facilitate internalization of the toxin via receptor mediated endocytosis. E. M. studies have shown that VT and its B subunit target the Golgi, RER and even the nuclear membrane following internalization. Treatment of Gb_3 containing cells with VT B subunit can induce apoptosis. Two proteins involved in the B cell differentiation, CD19 and the α2 interferon receptor, show sequence homology with the VT B subunit suggesting that Gb_3 binding is involved in their mechanism of action. We propose that it is the unique intracellular routing of Gb_3 via retrograde transport which mediates this role in signal transduction. Surface ligation of CD19 resulted in CD19 internalization to the nuclear membrane of Gb_3 positive but not Gb_3 negative human lymphoblastomas. Gb_3 is expressed on a variety of human tumours and we have suggested that VT can be used as an antineoplastic agent [1]. A series of human Gb_3 containing astrocytoma cell lines were tested for sensitivity to VT. Although all cells were sensitive, the sensitivity varied over a 5000-fold range despite approximately equivalent Gb_3 levels. Treatment of the least sensitive cell line with sodium butyrate initiated a 5000-fold increase in VT sensitivity (α2interferon sensitivity also increased) concomitant with an alteration in

intracellular VT targeting. In the most sensitive and in sodium butyrate treated cells, VT was internalized via retrograde transport to the nuclear membrane whereas VT resistant cells targeted the internalized toxin to another, as yet undefined (largely non lysosomal) intracellular location. These results indicate that intracellular routing can determine sensitivity to VT and that Gb_3 binding may provide an intracellular route from the cell surface to the nuclear membrane for Gb_3 binding proteins. We are investigating whether there are chemical differences in the Gb_3 of cells which target VT to the nucleus. The data suggests that there is an endosomal compartment in which Gb_3 is sorted for retrograde transport though Golgi-ER-nuclear membrane or to lysosomal or other intracellular vesicles. As a result of the pH sensitivity of fluorescein, use of FITC-VT B subunit to trace these intracellular pathways provides the first method for the direct measurement of the pH of these subcellular organelles.

1. Farkas-Himsley, Hill R, Rosen B, Arab S, Lingwood CA (1995) *Proc Natl Acad Sci* (in press).

S20. 11.35am

The Differentiation-Dependent Metabolism of Glyco(sphingo)lipids in HT-29 Cells is Controlled by Autophagy

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HT-29 cells are derived from human colon carcinoma and represent a suitable model to study the onset of enterocytic differentiation. Recently it was shown that the metabolism of both glycoproteins and glyco(sphingo)lipids is dramatically dependent upon the state of cell enterocytic differentiation. Furthermore, it was shown that the differentiated cells express an autophagic sequestration mechanism, controlled by a Gi3 protein. In order to correlate the metabolism of glyco(sphingo) lipids with the extent of autophagic sequestration, we have incubated tritium-labelled GM1 ganglioside and sphingosine with: (a) undifferentiated HT-29 cells; (b) differentiated HT-29 cells; (c) undifferentiated HT-29 cells, in the presence of asparagine (an inhibitor of autophagic degradation; (d) undifferentiated HT-29 cells, in the presence of pertussis toxin, which stabilizes the GDP-bound form of the Gi3 protein; and (e) undifferentiated HT-29 cells, transfected with a cDNA encoding the αi3 subunit of the Gi3 protein.

The results show that the extent of glyco(sphingo)lipid metabolism can be correlated with the rate of autophagic degradation in the different cell populations. Moreover, in undifferentiated cells, when autophagy is inhibited, the metabolism of glyco(sphingo)lipids resembles that expressed in differentiated cells. We conclude that autophagy plays an important role in the control of the differentiation-dependent metabolism of glyco(sphingo)lipids.

S20. 11.55am

Sphinogolipid Analogues and Membrane Traffic

S20 POSTERS

S20

Functional Requirement of Sphingolipids for Cell-Substratum Adhesion

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Sphingolipids that represent many glycosphingolipids and a unique phosphosphingolipid (sphingomyelin) occupy 5–10% of plasma membrane lipids. Here we investigated the physiological function of the sphingolipids using glycosphingolipid-deficient mouse melanoma cells. Both cell lines studied, MEB-4 and its mutant GM-95 which is deficient in glycosphingolipids, attached on extracellular matrices such as fibronectin, collagen, and laminin. Our finding indicates that glycosphingolipids do not directly contribute to the cell-substratum adhesion. In GM-95, the amount of sphingomyelin as a sole sphingolipid increased. As a result, the total amount of sphingolipids was constant between the two cell lines. Removal of plasma membrane sphingomyelin with neutral sphingomyelinase treatment on GM-95 cells strongly inhibited cell adhesion. The inhibition was also observed by metabolic inhibitors of sphingolipid synthesis. These observations indicate that the absence of sphingolipids on the plasma membrane resulted in cell detachment from extracellular matrices. In contrast to other lipids, sphingolipids were characteristically concentrated at the focal contact of both cells. Furthermore, the level of tyrosine phosphorylation was greatly reduced by neutral sphingomyelinase treatment on GM-95 cells, but not on MEB-4 cells. In conclusion, sphingolipids clustered on plasma membrane constitute the adhesion machinery as an essential component.

S20

The Pattern and Concentration of Glycosphingolipids in Spermatozoa Membrane – a Parameter for the Evaluation of Spermatozoa Membrane Fragility in Freezing-Thawing Processes

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Ganglioside GM4 and galactosyl-ceramide-I^3-sulfate have been isolated from the membranes of cock spermatozoa. Their concentration has been found to be 404 ± 15 nmol and 585 ± 82 nmol per g of wet spermatozoa, respectively. Ganglioside GM4 constituted about two thirds of total ganglioside-bound sialic acid, other gangliosides that were identified in the membrane of cock spermatozoa being GD3, GD1b and GT1b.

Total ganglioside-bound sialic acid in the membrane of boar sperm cells has been estimated to be 608 ± 55 nmol per g of wet cells. The following distribution of lipid-bound sialic acid was found in the gangliosides of boar spermatozoa membranes: GM1a (9%), Fuc-GM1a (34%), GD1a (29%), GQ1b (28%).

S20

Variability in Testis Ganglioside Content and Composition as a Reference for Elucidation of Spermatozoa Production

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Concentration of ganglioside-bound sialic acid as well as the ratio between sialic acid of monosialo- and sialic acid of polysialogangliosides have been determined in testis using the following methods: extraction of the tissue with chloroform-methanol, Folch partition, chromatography on DEAE-Sephadex A-25 and resorcinol colorimetric reaction. The efficiency of ion-exchange chromatography has been monitored by HPTLC. The following resullts have been obtained, in μg per g wet tissue (in parentheses is the ratio between sialic acid of monosialo- and sialic acid of polysialogangliosides): ram 90 ± 2.5 (1:8), boar 3.99 ± 0.07 (1:5.19), turkey 43.4 (1:1.65), cock 33.14 (1:1.43), *Streptopelia decaocto* 61.8 ± 3.65 (1:0.8).

S20

Cloned β1,4 *N*-Acetylgalactosaminyltransferase (GM2 Synthase): Subcellular Localization, Formation of Disulfide Bonded Species, and Release of a Soluble Form

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To define the properties of GM2 synthase, we stably transfected CHO cells with three myc epitope-tagged forms of the cloned human GM2 synthase gene: the native enzyme; the lumenal domain of GM2 synthase fused to the cytoplasmic and transmembrane domains of *N*-acetylglucosaminyltransferase I (GNT); and the transmembrane and lumenal domains of GM2 synthase fused to the cytoplasmic domain of the lip33 form of human invariant chain. Whereas the native enzyme and the GNT form were found in the Golgi apparatus by immunofluorescence and immunoEM, the Iip33 form was restricted to the ER. Cells transfected with all three constructs contained high levels of GM2 synthase activity *in vitro*, but only the native enzyme and the GNT form were able to synthesize glycolipid product *in vivo*. The enzyme produced by all three constructs was present in the transfected cells in a disulfide bonded form, which may be a homodimer. Greater than 90% of the total activity of the native enzyme was released into the culture medium as a soluble, disulfide bonded form. In contrast 68% of the GNT-GalNAcT/myc form was released, but no activity could be detected in the medium of cells transfected with the Iip33 form. These results suggest that formation of disulfide bonded species of GM2 synthase may occur in the ER while release of a soluble form of the enzyme may occur later in the secretory pathway.

S20

Metabolism of a Fluorescent Ceramide Analogue ($LRh-C_{12}$-Cer) in Human Fibroblasts

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In the present study we describe the metabolic behaviour of a ceramide analogue, bearing the fluorophore Lissamine-Rhodamine ($LRh-C_{12}$-Cer), in human skin fibroblasts, and the effect of Brefeldin A (BFA) on fluorescent sphingomyelin (SM) synthesis as well as its intracellular transport. $LRh-C_{12}$-Cer, incorporated into egg-PC liposomes, was taken up by cells in a dose and time dependent manner. $LRh-C_{12}$-SM was the only fluorescent derivative recovered both after 24 h Pulse and 24 h Pulse/24 h Chase experiments. Most of the SM was found in the culture medium, while less than 10% of the total amount was cell associated, indicating that SM is translocated from the Golgi apparatus to the cell surface where it is released into the medium.

The presence of 5 μg ml^{-1} BFA results in a 85% decrease of SM recovered in the medium with a corresponding five-fold increase of the cell associate SM. These results support the idea that in human fibroblasts the passage to the plasma membrane of the newly synthesized SM depends on vesicular transport.

After the BFA removal, several hours were required to restore the normal cell content of fluorescent SM. In addition, preliminary data indicate that during the BFA induced intracellular SM accumulation, the SM synthesis is inhibited, and only after the release of the SM bulk into the medium, a net SM synthesis again becomes detectable.

In conclusion, $LRh-C_{12}$-Cer proves to be a useful tool for studying the SM metabolism and trafficking in human skin fibroblasts.

S21. DIFFERENTIATION-DEPENDENT, CELL TYPE-SPECIFIC CARBOHYDRATE MARKERS

Chairs: Ikuo Yamashina, Kenneth Lloyd

S21. 9.50am

Use of Monoclonal Antibodies in the Studies of Cellular Carbohydrate Markers

I. Yamashina
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Glycoproteins and glycolipids are often specific markers associated with specific cell types and are regarded as having correlations with cell functions including cell growth differentiation. Studies along these lines have been made mainly on glycolipids in blood cells. On differentiation the expression pattern of glycolipids changes dramatically. We have carried out

similar studies on glycoproteins, especially mucin-type glycoproteins. Using monoclonal antibodies directed towards mucin glycoproteins, we have found that unique epitopic structures occur on cancer cell surfaces, which include Tn, sialyl-Tn and sialyl-Le^a type antigens. Of the cell lines, such as Jurkat, K 562 and HL 60 examined, only Jurkat expresses Tn as prosthetic group of leukosialin. K 562 expresses GalNAc-Ser/Thr, but does not form Tn antigen due to the absence of the cluster structure. It should be noted that HL 60 cells possess GM3 as the major glycolipid antigen which appears to correspond to sialylated T-antigen in mucin. We have also found that sialyl-Le^a antigens are abundant on cancer cell surfaces and these antigens constitute mucin O-glycans of large molecular size. This finding seems to be correlated with the expression of embryoglycan on embryonic cell surfaces.

Mechanism of expression of the cellular carbohydrate markers is worthy of discussion, since the factors which regulate the expression should be multiple and glycosyltransferase is only one of the factors.

S21. 10.15am

Cell Type-Specific Expression of Gangliosides in Primary Cultures of Rat Cerebellum

I. Kawashima[1], I. Nagata[2], and *T. Tai*[1]
[1] *The Tokyo Metropolitan Institute of Medical Science, Honkomagome, Bunkyo-ku, Tokyo 113, Japan.*
[2] *The Tokyo Metropolitan Institute of Neuroscience, Musashidai, Fuchu, Tokyo 183, Japan.*

We studied the expression of gangliosides on neurons and glia in rat primary cerebellar cultures using an immunocytochemical technique with mouse monoclonal antibodies (MAbs) to various gangliosides. Twenty-two MAbs that specifically recognize an individual ganglioside were used. Our study revealed that there is a cell type-specific expression of gangliosides in the primary cultures. GD1b was expressed in the granule cells and another type of small neurons. An *O*-Ac-disialoganglioside, which was suggested to be *O*-Ac-LD1, was restrictedly detected in Purkinje cells. GD2 was specifically present in the type of small neurons, but not in the granule cells. On the other hand, GM4 was specifically expressed in the astrocytes, but not in any other types of cells including the oligodendrocytes. GD3 was associated not only with the granule cells, but also with both astrocytes and oligodendrocytes. The other gangliosides were not detected clearly in these cells. These results suggest that a number of gangliosides may be useful markers for primary cultures of the rat cerebellum; particularly GD1b, *O*-Ac-LD1, and GM4 for the granule cells, Purkinje cells, and the astrocytes, respectively.

S21. 10.30am

CD15 Shows a Compartmentation of the Mouse Developing Forebrain

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Institute of Neuroanatomy, H.-Heine-Universität, Moorenstr. 5, D 40001-Düsseldorf, Germany.

CD15 monoclonal antibodies define the trisaccharide $3(\alpha)$-fucosyl-*N*-acetyl-lactosamine or CD15 epitope. The spatial and temporal expression of this epitope was investigated by immunohistochemistry in the brain of postimplantation mouse embryos. Immunoreactivity (IR) is first detected at around embryonic day 7 (E7), labelling the whole neuroectodermal layer. After closure of the neural tube at E9, the expression is abruptly reduced and can no longer be detected in the CNS until midgestation time. Beginning at late E11, CD15 becomes re-expressed. It is first found accumulated at facing areas of the ventricular and pial surfaces, which later become interconnected by radially oriented processes. About 3 days later (E14), these can be clearly ascribed to radial glial fibres, spanning the wall of the CNS. Their spatial distribution along the ventricular surface of the forebrain is confined and marks regional fields, identical to those described as neuromeric boundaries within the prosencephalon. The basic organization of the mammalian forebrain can thus, for the first time, be correlated with the location and orientation of radial glial fibres responsible for the translocation of neuroblasts to their settling regions.

In parallel to the expression on radial glial cells and their processes the carbohydrate epitope is also localized perisomatically on clusters of neurons situated in close topographic relation to evaginations of the neuroepithelial wall. Their location suggests that CD15 expression is associated here with the organization of tissue translocation in the developing forebrain.

Between E17 and the end of the embryonic period the transformation of radial glial cells into regular astrocytes can be followed by the changing CD15 expression pattern. Western blots from CNS samples obtained between E14 and birth shows a single band of CD15 glycosylated protein with an approximate molecular weight of 24 kDa.

S21. 10.45am

Antibody Directed to Le^y Inhibits Blastocyst Implantation in the Mouse

B. A. Fenderson[1] and Z. Zhu[2]
[1] *Department of Pathology, Anatomy, and Cell Biology, Thomas Jefferson University, Philadelphia, USA.*
[2] *Department of Biochemistry, Dalian Medical University, Dalian, Peoples Republic of China.*

Le^y is present on the surface of the mouse blastocyst and uterine epithelium. This oligosaccharide is carried on many uterine glycoproteins, however new species appear on Day 4 coincident with the onset of uterine receptivity. The function of Le^y in implantation was tested by injecting monoclonal antibody directly into the uterine lumen. The effects of intrauterine injections on implantation were scored by comparing the number of viable embryos to the number of corpora lutea on Day 10. Injection of purified anti-Le^y IgM into the uterine lumen on the afternoon of Day 4 significantly inhibited implantation. This effect was dose-dependent and obtained during a narrow time-window. Inhibition of implantation was not observed in contra-lateral uterine horns injected with saline, nor was it observed in uterine horns injected with other anti-carbohydrate antibodies. We conclude that binding of anti-Le^y to the blastocyst or luminal epithelium masks a ligand involved in cell adhesion during an early step in implantation.

This investigation received support from the Special Programme of Research, Development, and Research Training in Human Reproduction, World Health Organization.

S21. 11.00am

7-*O*-acetyl G_{D3}, a carbohydrate activation antigen of human T cells

Bernhard Kniep[1], Christine Claus[2], Jasna Peter-Katalinic[3], and Manfred Nimtz[1]
[1] *GBF-National Research Centre for Biotechnology, Braunschweig, Germany.*
[2] *1st Medical Hospital of the University of Mainz, Mainz, Germany.*
[3] *University of Bonn, Germany.*

We found that the monoclonal antibody U5 strongly bound to an alkali labile form of ganglioside G_{D3} in the disialoganglioside fractions from both human leukocytes and bovine buttermilk. Antibodies to 9-*O*-acetyl G_{D3} failed to bind to this labile G_{D3} and antibody U5 failed to bind to 9-*O*-acetyl G_{D3}. Our interest in the structural characterization of the U5 antigen came from the observation that binding of mAb U5 to human T-cells induced – in contrast to that of several 9-*O*-acetyl G_{D3} specific mAbs – a strong proliferation of human T cells, suggesting that the U5 antigen was a new carbohydrate activation antigen. Here we describe the purification of the U5 antigen and present evidence that this antigen is the ganglioside 7-*O*-acetyl G_{D3}. The antibody showed a weak cross reactivity to non *O*-acetylated G_{D3} but it bound to at least 15 fold lesser amounts of 7-*O*-acetyl G_{D3}. After immunoprecipitation of solubilized human T-cells by mAb U5, and analysis of the chloroform-methanol extract, it was found that 7-*O*-acetyl G_{D3} was the only precipitated lipid component.

S21. 11.15am

A Novel Glucuronyltransferase Associated with the Biosynthesis of HNK-1 Epitope on Neural Cell Adhesion Molecules

K. Terayama, T. Seiki, S. Oka and *T. Kawasaki*
Department of Biological Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606-01, Japan.

The HNK-1 epitope, which is expressed specifically on neural cell adhesion molecules such as N-CAM, L1 and J1 as well as on specific neural glycolipids, is presumed to play important roles in cell-cell interactions and recognitions in the nervous system. The epitope is characterized as a sulfated glucuronyl residue, a very unusual carbohydrate structure. In order to study the mechanisms of the temporally and spatially regulated expression of the epitope during the development of the nervous system, we tried to isolate and characterize glucuronyltransferase (GlcAT) which is a key enzyme for the biosynthesis of this unique epitope. Rat brain GlcAT was separated into two groups: one (GlcAT-L) utilizes glycolipids and the other (GlcAT-P) utilizes proteins as acceptors [1], suggesting that the expression of glycolipid and glycoprotein epitopes can be regulated independently during the development. A GlcAT-P was purified to homogeneity (45 kDa on SDS-PAGE) from the NP-40 extract of rat brain by means of various types of chromatography including an affinity column of asialoorosomucoid. The purified enzyme transferred GlcA from UDP-GlcA to Galβ1-4GlcNAc structure on glycoproteins but not to the same structure on glycolipids. Biochemical and enzymological studies demonstrated that the enzyme is a novel type of glucuronyltransferase which has not been described before.

1. Oka S, Terayama K, Kawashima C, Kawasaki T (1992) *J Biol Chem* **267**: 22711–14.

S21. 11.30am

Comparison of O-Linked Carbohydrate Chains in MUC-1 from Normal Breast Epithelium Cell Lines and Breast Cancer Cell Lines

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Imperial Cancer Research Fund, London, UK.

Many anti-MUC-1 monoclonal antibodies (mAb) are directed against the tandem repeat peptide region of this mucin. MAbs that react preferentially with tumours are thought to do so because of the selective exposure of peptide epitopes due to underglycosylation of MUC-1 in cancer cells. We have examined this hypothesis by analysing the carbohydrate chains of MUC-1 immunoprecipitated by CT-1 antiserum from [3-H]GlcN-labelled SV-40 transformed normal epithelial cell lines (MTSV1-7 and HB-2) and three breast cancer cell lines (MCF-7, BT-20 and T47D). Analysis by HPAEC of the ratio of GlcN/GalN incorporated into MUC-1 showed that the normal cell lines had higher ratios than did the cancer cell lines, with T47D having the lowest ratio. A detailed structural analysis of the O-linked chains was carried out with MUC-1 from HB-2 and T47D. MUC-1 from T47D had rather a simple glycosylation pattern with NeuAcα2 $\rightarrow$ 3Galβ1 $\rightarrow$ 3GalNAc-ol, Gal-β1 $\rightarrow$ 3GalNAc-ol, and GalNAc-ol predominating. MUC-1 from HB-2 cells had more complex structures including a number of disialo species (Neuα2 $\rightarrow$ 3Galβ1 $\rightarrow$ 3[NeuAc-α2 $\rightarrow$ 6]GalNAc-ol, NeuAcα2 $\rightarrow$ 3Galβ1 $\rightarrow$ 4GlcNAcβ1 $\rightarrow$ 6 [NeuAcα2 $\rightarrow$ 3Galβ1 $\rightarrow$ 3]GalNAc-ol and a larger disialo-GlcNAc-containing species) and their unsialylated counterparts. We conclude that MUC-1 from breast cancer cell lines have simpler, less sialylated carbohydrate chains than MUC-1 from normal breast epithelium cell lines, although there was considerable variation among the three tumour cell lines examined. These differences may explain the differential reactivity of some anti-MUC-1 mAbs, although the degree of substitution of Ser/Thr may also play a role.

S21 POSTERS

S21

Production of a Monoclonal Antibody Specific for Phosphonoglycosphingolipid F-21

S. Araki, S. Abe and M. Satake
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Gangliosides have not been found so far in the mollusca, *Aplysia kurodai*. Instead, *Aplysia* has glycolipids containing 2-aminoethylphosphonate (2-AEPn) which belong to a new family of phosphonoglycosphingolipids (PnGSLs).

PnGSL F-21 was found specifically in the nervous system of *Aplysia* and its chemical structure was determined as 3-*O*-MeGalβ1 $\rightarrow$ 3(2-AEPn $\rightarrow$ 6)GalNAcα1 $\rightarrow$ 3[6′-*O*-(2-AEPn) Galα1 $\rightarrow$ 2](2-AEPn $\rightarrow$ 6)Galβ1 $\rightarrow$ 4Glcβ1 $\rightarrow$ 1Cer [1].

We generated a monoclonal antibody, termed mAb 4E6, using F-21 as the immunogen. The specificity of the monoclonal antibody was mainly defined by the TLC immunostaining method. MAb 4E6 was found to react only with F-21, but not with any other PnGSLs, SGL-II and SGL-I′, structurally related to F-21, or SGL-I. These results indicate that mAb 4E6 specifically recognizes the 3-*O*-MeGalβ1 → 3(2-AEPn → 6) GalNAc structure as epitope. Immunohistochemical studies on the cellular localization of F-21 will be reported.

1. Abe S, Araki S, Satake M, Fujiwara N, Kon K, Ando S (1991) *J Biol Chem* **266**: 9939–43.

S21

GM2 Ganglioside Expressed on Rheumatoid Arthritis Synovia

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Rheumatoid arthritis (RA) is an autoimmune disease, not a tumourous lesion, but its synovia proliferate in a tumour-like manner. The author analysed gangliosides of 40 cases of RA synovia, and detected GM2 ganglioside as one of the main gangliosides of RA synovia. GM2 ganglioside is considered a malignant tumour marker, however RA is not a tumour, so the author speculates that GM2 ganglioside is a proliferation marker of RA synovia. RA synovia were sampled from 40 cases, at the time of total knee arthroplasty. Total wet weight of the synovia was 700 g, the tissues were extracted by Folch's method. Lipid contamination was removed by successive acetone partition, and finally by phenyl boronate agarose PBA60 (Amicon Corp.) column chromatography. The glycosphingolipid mixture was developed on an HPTLC plate, gangliosides were detected by resorcinol-reagent and GM2 was detected by enzyme-immunostaining, using an anti-asialoGM2 antibody. The most dominant gangliosides of RA synovia are GM3 and GD3, followed by GM2. GM2 ganglioside is not detected in benign tissues, but RA synovia express GM2, so the author speculates GM2 as a proliferation marker of RA, possibly related to an apoptosis disorder in RA synovia.

S21

Ganglioside Antigen of DU-PAN-2 in a Human Pancreatic Cancer

Y. Hamanaka[1], *S. Hamanaka*[2], F. Inagaki[3], M. Suzuki[3], M. Abe[4], and A. Suzuki[3]
[1]*Yamaguchi University School of Medicine, Japan.*
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[3]*The Tokyo Metropolitan Institute of Medical Science, Japan.*
[4]*Tokyo Bunka Med. Tech., Tokyo, Japan.*

Gangliosides were isolated from the tumour of a patient with pancreatic cancer (duct cell carcinoma) and Lewis negative blood phenotype and the structures of DU-PAN-2 reactive gangliosides were analysed. The reactive gangliosides were NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer containing normal and hydroxy fatty acids. TLC-immunostaining and ELISA with chemically synthesized gangliosides demonstrated that DU-PAN-2 reacted strongly with $IV^3\alpha$NeuAc-Lc_4Cer, very weakly with $IV^3\alpha$NeuAc-nLc_4Cer, and moderately with $IV^6\alpha$NeuAc-Lc_4Cer and $IV^6\alpha$NeuAc-nLc_4Cer. Thus, the DU-PAN-2 reactive gangliosides in the tumour are concluded to be $IV^3\alpha$NeuAc-Lc_4Cer, and DU-PAN-2 has a rather broad specificity.

Gangliosides extracted from serum of the same patient did not contain a detectable amount of DU-PAN-2 reactive gangliosides on TLC-immunostaining but glycoprotein bands at high molecular mass region were detected by Western blotting.

S21

A Monoclonal Antibody to the Gastric Gland Mucous Cell-Derived Mucin, HIK-1083, Reacts with Oligosaccharides Bearing Peripheral α-Linked GlcNAc Residues

K. Ishihara[1], M. Kurihara[3], Y. Goso[2], T. Urata[2] and K. Hotta[2]
Departments of [1]*Chemistry, and* [2]*Biochemistry, Kitasato University School of Medicine, Sagamihara, Japan.*
[3]*Isehara Research Laboratory, Kanto Chemical Co. Inc., Isehara, Japan.*

Mammalian gastric gland mucous cells (GMC), including mucous neck and pyloric gland cells, synthesize and secrete mucin species, which can be detected by the paradoxical concanavalin A staining method [1]. We established a monoclonal antibody (MAb), HIK-1083, which specifically recognizes the mucin derived from rat gastric GMC using immunochemical and immunohistochemical methods. The aim of this study was to determine the specific epitope structure of this MAb. For this purpose, mucins of the deep mucosal layer of rat stomach and that from pig gastric fundic mucosa were purified using Bio-Gel A-1.5 m column chromatography followed by CsCl equilibrium density gradient centrifugation. The oligosaccharides obtained from the purified mucin by alkaline borohydride reduction inhibited the reaction of the MAb with the purified mucin on the ELISA plate. An active oligosaccharide peak fractionated on a column of Bio-Gel P-6 near the maltohexaose standard was pooled and then separated by HPLC (TSKgel ODS80TM). Two purified oligosaccharides, OP-2 and OP-5, which indicated the following carbohydrate composition reacted with HIK-1083 in the competitive ELISA. (OP-2, GalNAc-ol:Gal:GlcNAc = 1:2:3; OP-5, GalNAc-ol:Gal:GlcNAc:Fuc = 1:2:2:1) The ^{1}H- and ^{13}C-NMR spectra of OP-2 and OP-5 showed that these oligosaccharides have peripheral α-linked GlcNAc residues, and the profiles of the ^{1}H-NMR spectra coincided exactly with that reported by Halbeek *et al.* [2]. In the paranitrophenyl (PNP) derivatives of monosaccharides, only PNP-α-GlcNAc reacted with HIK-1083. These results indicate that this Mab recognizes a particular carbohydrate structure which might be specifically involved in the mucin derived from the gland mucous cells of mammalian gastrointestinal mucosa.

1. (1978) *J Histochem Cytochem* **26**: 233.
2. Halbeek *et al.* (1983) *Biochim Biophys Acta* **747**: 107.

S21

Production and Characterisation of Monoclonal Antibodies to Human Salivary Mucins

P. AA. Nielsen, U. Mandel, M. H. Therkildsen and H. Clausen
Department of Oral Diagnostics, Faculty of Health Sciences, School of Dentistry, University of Copenhagen, Denmark.

Two distinct mucin components, MG1 and MG2, have previously been identified based on their molecular weight in

submandibular-sublingual saliva. With the aim of characterizing the expression pattern of these mucins, we have prepared monoclonal antibodies (MAbs) directed against the peptide backbone of purified, partially deglycosylated MG1, and against a synthetic peptide derived from MG2 (MUC7). MAb PANH2 was raised against MG1, and was found to react with glycosylated, partially deglycosylated and fully deglycosylated mucin by Western blot. PANH3 was generated to a N-terminal peptide derived from MUC7 (MG2), and stained by Western blot a narrow smear of approx 150–250 kDa from untreated saliva, and a lower molecular weight smear of TFMSA treated saliva. Using PANH2 and PANH3 in immunohistology of frozen sections of human submandibular, sublingual, parotid and small salivary glands, a differentiated cell staining pattern was found. PANH2 defining MG1 specifically labelled all mucous cells, whereas PANH3 defining MG2 appeared to label subpopulations of serous cells. Double immunofluorescence staining with PANH2 and PANH3 showed an exclusive non-overlapping staining pattern.

The development of these antibody probes will be useful as markers of salivary mucins and to define cell types in pathological disorders in salivary glands.

S21

A New Monoclonal Anti-Lewis b Antibody that Binds to Human Bronchial Mucins

N. Emery[1], S. B. Palfai[3], R. Oriol[2], G. Place[3], R. Hall[3], M. Lhermitte[1] and *P. Roussel*[1]
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[2]*Inserm No 78, Av. Paul-Vaillant Couturier, 98407 Villejuif, France.*
[3]*Bayer UK Ltd, Pharma Research, Stoke Court, Stoke Poges, SL2 4LY, UK.*

A monoclonal antibody (3D3) was generated against human bronchial mucins. The 3D3 MAb stained specifically bronchial goblet cells of the surface and mucous cells of the submucosal glands. Mucins treated with fucosidase did not react with the 3D3 MAb. Adsorption experiments performed with carrier-bound oligosaccharides (Synsorbs) demonstrated that the antibody was a member of the anti-Lewis b antibodies family. This 3D3 MAb cross-reacted with Lewis a and Lewis Y structures, but not with H structures. Strong anti-Lewis b MAb are difficult to obtain, and they cross-react with either Lewis a (anti-Le^{bL}) or H (anti-Le^{bH}) [1]. This 3D3 MAb, which reacts with Lewis Y epitopes, is slightly different from the anti-Lewis antibodies described so far, since it does not belong to the anti-Le^{bL} or the anti-Le^{bH} families.

The 3D3 MAb has been tested on respiratory mucins secreted by patients with different blood group and secretor status. It binds to mucins from secretor (Se) and Lewis (Le) individuals, without any significant difference. Its affinity for mucins from Lewis (Le) but non secretor (se) individuals is lower, and lower still when mucins are highly sialylated. As expected, mucins from secretors (Se) but non Lewis (le) individuals are not recognized by the 3D3 MAb.

These data suggest that the 3D3 MAb may be used to monitor the fucosylation of mucins secreted by human bronchial epithelial cells in culture.

1. Good *et al.* (1992), *Vox Sang* **62**: 180–89.

TUESDAY 24 AUGUST, MORNING

PLENARY LECTURES

S22. 8.00am

New Classes of Sialic Acid, Sialidase, and Polysialic Acid

Y. Inoue
Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, Japan.

In 1978, S. Inoue found the first example of polysialic acid structure in animal origin [1]. Following her discovery in trout eggs an increasing number of $\alpha2 \rightarrow 8$-linked polySia chains have been found in various animal species including eel, frog, chicken, rat, and humans. Most of the polySia chains appear to be expressed in a spatio-temporally regulated manner and are considered to be a unique class of oncodevelopmental glycotopes. In 1986, we uncovered a new sialic acid, KDN (= 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid; deaminated neuraminic acid) residue. The presence of various types of KDN linkages have been identified in bacterial capsular polysaccharides, glycoproteins and glycosphingolipids of a wide variety of animal cells and tissues. KDN thus can be regarded to constitute a new class of sialic acids in nature. More recently, we have found a series of enzymes responsible for biosynthesis and degradation of KDN-glycan chains: CMP-KDN synthetase, KDN-transferases, and KDNase. We have also demonstrated the natural occurrence of multiple forms of polySia structures such as: (a) the different forms of $\alpha2 \rightarrow 8$-linked homo- and heteropolymers of Neu5Ac and Neu5Gc with or without *O*-acetyl substituents; (b) $\alpha2 \rightarrow 8$-linked polyKDN; and (c) $(\rightarrow 5\text{-}O_{glycolyl}\text{-Neu5Gc}\alpha2\rightarrow)_n$.

In this Symposium we shall discuss the most recent results of our studies on; (a) identification of different types of chain termination signals for *in vivo* polysialylation; (b) identification of a possible polysialylation signal in the N-CAM core glycan chain; and (c) a possible functional role of (KDN)GM3-Gg3 interaction as a basic mechanism for gamete adhesion.

1. Inoue S (1978) *BBRC* **83**: 1018–23.

S23. 8.30am

The Sialoadhesin Family of Cellular Interaction Molecules

P. R. Crocker[1], S. Freeman[1], S. Mucklow[1], A Hartnell[1] and S. Kelm[2]
[1]*Imperial Cancer Research Fund Laboratories, Institute of Molecular Medicine, Oxford University, Oxford, UK.*
[2]*Biochemisches Institut, University of Kiel, Germany.*

Sialoadhesin is a macrophage-restricted receptor of 185 kDa

that mediates sialic acid-dependent binding to cells via recognition of NeuAcα2,3Gal in N- and O-glycans and in gangliosides. Recent cDNA cloning revealed that sialoadhesin is a new member of the immunoglobulin superfamily (IgSF) with 17 immunoglobulin-like domains, the largest number so far described for a cell surface member of this superfamily. Sialoadhesin is structurally related to a distinct subgroup of the IgSF that includes CD22, the myelin associated glycoprotein (MAG), the avian Schwann cell myelin protein (SMP) and CD33. These proteins share a homologous N-terminal V-like domain and variable numbers of C2-like domains with about 50% sequence similarity over the N-terminal 2-4 Ig-like domains. Since earlier work of others showed that CD22 could function as a sialic acid-dependent adhesion molecule, we have investigated whether the other mammalian proteins related to sialoadhesin can also function in sialic acid-dependent adhesion. A range of cell binding assays were carried out with recombinant Fc-chimaeric forms of sialoadhesin, CD22, MAG and CD33 as well as with COS cells that were transiently transfected with the respective full-length cDNAs. Our results show that these proteins can mediate sialic acid-dependent adhesion, but with distinct specificities. However, whilst sialoadhesin, CD22 and MAG could mediate binding when expressed in COS cells, CD33 was unable to do so, unless the transfected cells were pretreated with sialidase. These results raise the possibility that binding mediated by these lectins can be modulated by interactions with endogenous ligands and may provide an explanation for why sialoadhesin has evolved such an extraordinary number of Ig-like domains.

S24. 9.00am

Multivalent Lectin-Carbohydrate Interactions. A New Dimension of Binding Specificity

C. F. Brewer

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Lectin binding to the surface of cells leads to cross-linking of glycoconjugate receptors, including glycoproteins and glycolipids, which, in many cases, is related to a variety of biological signal transduction processes. We have observed that many naturally occurring cell surface oligosaccharides are multivalent and capable of binding and precipitating with specific lectins [1]. These cross-linking interactions lead to a new source of binding specificity: namely, the formation of homopolymeric carbohydrate-lectin cross-linked lattices, even in the presence of mixtures of the molecules [2]. Our recent studies show that many of these lattices are crystalline, and that structural information can be obtained using electron microscopy and X-ray diffraction techniques. For example, the tetrameric GalNAc/Gal specific lectin from *Glycine max* (soybean agglutinin) forms crystalline cross-linked lattices with a series of branched chained oligosaccharides containing terminal *N*-acetyllactosamine residues. We will describe the results of electron microscopy and X-ray crystallographic studies of these complexes which provide insight into the structure-function properties of multivalent lectins and carbohydrates.

1. Bhattacharyya L, Ceccarini C, Lorenzini R, Brewer CF (1987) *J Biol Chem* **262**: 1288–93.
2. Bhattacharyya L, Brewer CF (1992) *Eur J Biochem* **208**: 179–85.

S22. SIALIC ACID, SIALIDASE AND POLYSIALIC ACID

Supported by Taiyo Kagaku Co.

Chairs: Rick Troy, Roland Schauer

S22. 9.50am

Localization of Sialic Acid *O*-Acetylation in Rat Liver Hepatocytes

R. Chammas, J. M. McCaffrey, A. Klein, M. G. Farquhar and A. Varki

Glycobiology Program, Cancer Center and Division of Cellular and Molecular Medicine, University of California, San Diego, CA, USA.

Sialic acids (Sia) are a family of negatively charged sugars, usually found as terminal monosaccharides in animal glycans. *O*-acetylation of Sia at the 4, 7, 8 and 9 positions are among the commonest modifications studied so far and have been implicated in the binding of viruses, modulation of complement activation, and embryonic development. Rat liver hepatocytes are rich in 9-*O*-acetylated membrane sialoglycoproteins and comprise an useful model for studies of biosynthesis of this modification. Cytosol-free Golgi preparations from rat liver were used for studies of the biosynthetic activity of individual Golgi compartments. Usage of CMP-Sia-FITC as a donor for Sia allowed detection of newly synthesized sialoglycoconjugates immunochemically. Sia-FITC and [^{3}H]-Gal were incorporated into the very same glycoconjugates, as determined by immunodepletion assays, indicating an overlap of galactosyltransferase and sialyltransferase machineries in the same Golgi compartment. On the other hand, Sia-FITC and [^{3}H]-acetate were never found in the same molecules. At the ultrastructural level, Sia-FITC uptake was studied with anti-FITC antibodies, and 9-*O*-acetylated-Sia was probed with an Influenza C hemagglutinin-esterase derived molecule [1]. Analysis of Golgi preparations with these probes showed a segregation of newly synthesized sialoglycoconjugates from 9-*O*-acetylated-sialoglycoconjugates present in a post-Golgi compartment. Taken together, these results suggest that Sia *O*-acetylation is a late event in the exocytic pathway. Its precise localization in post-Golgi compartments is now under investigation.

1. Klein *et al.* (1994) *Proc Natl Acad Sci USA* **91**: 7782.

S22. 10.15am

Primary Structure of CMP-*N*-Acetylneuraminic Acid Hydroxylase from Pig Submandibular Glands

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cDNA encoding CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac) hydroxylase [EC. 1.14.13.45], which is the key enzyme in the biosynthesis of *N*-glycolylneuraminic acid-containing glycoconjugates, has been cloned. Information obtained by partial sequence analysis of purified CMP-Neu5Ac hydroxylase from pig submandibular glands [1] was used to design short degenerate primers for use in polymerase chain reactions with pig submandibular gland cDNA templates. A 96 bp specific cDNA fragment was amplified and used to isolate two clones from a pig submandibular gland cDNA library. The cloned cDNAs (clone 1: 1.8 kbp, clone 2: 1.1 kbp) exhibited the same open reading frame containing all sequence information obtained by partial sequencing of the purified CMP-Neu5Ac hydroxylase, while polyadenylation was observed at different sites in the non-coding region. The missing 5′-region of the cDNA was obtained by 5′-RACE and contained an ATG in a KOZAC consensus sequence. Furthermore, Northern blot analysis led to the assumption that the full length cDNA of the CMP-Neu5Ac hydroxylase had been cloned. Regions of the deduced amino acid sequence exhibited considerable homology to peptide sequences obtained from the purified mouse hydroxylase. Otherwise, the primary sequence of the CMP-Neu5Ac hydroxylase exhibited no significant homologies to any known protein.

1. Schlenzka W, Shaw L, Schneckenburger P, Schauer R (1994) *Glycobiology* **4**: 675–83.

S22. 10.30am

Genomic Organization and Expression of the Rat Cytosolic Sialidase Gene

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We have previously cloned a cDNA of a rat cytosolic sialidase which is strongly expressed in skeletal muscle. Both the highest enzyme activity as well as the highest mRNA level are present in this tissue. To understand the basis of the expresion of this sialidase, we have cloned and sequenced the rat gene and its 5′-upstream region from a rat genomic library. The gene encoding the 1.8 kb skeletal muscle mRNA was found to span 3.4 kb of genomic DNA and to consist of two introns and three exons. Exon 1 contains the 5′ non-coding region, and exons 2 and 3 encode the regions containing the AUG initiation codon and two ASP-boxes, respectively. In the 5′-upstream sequence, there is a TATA box and two E-box pairs known as consensus binding sites for muscle specific transcription factors. Analysis of the expression of transfected sialidase enhancer/promotor expression plasmid demonstrated the sialidase enhancer/promotor to be active in rat L6 myogenic cells shown to express this gene but inactive in rat 3Y1 fibroblasts shown not to express the enzyme. The transcription activity was increased three-fold after induction of myoblast differentiation. The L6 cells, in fact, elevated the levels of the mRNA and the enzyme activity concomitantly with myotube formation immediately after myogenin expression. Interestingly, in contrast to the sialidase, the cells then showed a tendency to reduce the mRNA level of the α2-3Galβ1,3GlcNAc sialyltransferase which is also highly expressed in skeletal muscle and produces the sialyl linkages suitable for the sialidase substrate. These observations give an account of constitutive expression of the sialidase gene in skeletal muscle.

S22. 10.45am

Occurrence of α2,8 Polydeaminoneuraminic Acid in Mammalian Tissues: Widespread and Developmentally-Regulated but Highly Selective Expression on Glycoproteins

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Mono- and polymeric deaminated *N*-acetylneuraminic acid, KDN (2-keto-3-deoxy-D-*glycero*-D-*galacto*-nonulosonic acid), was recently detected in rainbow trout egg polysialoglycoproteins. We have found α2,8 poly KDN bearing glycoproteins by immunohistochemistry and Western blot analysis in various mammalian tissues with the use of the mouse monoclonal antibody mAb kdn8kdn recognizing this glycan with a degree of polymerization of ⩾3. Immunoreactivity was abolished by treatment with a bacterial KDNase specifically hydrolysing deaminoneuraminyl ketosidic linkages. The α2,8 poly KDN glycan was found in almost all studied embryonic and adult tissues. It showed a developmentally regulated expression in kidney, brain, lung and muscle. Strikingly, by Western blotting a prominent single band of 150 kDa was detectable in all tissues except for a ⩾350 kDa band in kidney. The ubiquitous yet selective expression may be indicative of a general function of the α2,8 poly KDN-bearing glycoprotein.

S22. 11.00am

Purification and Characterization of a Unique α2,8-Polysialylated Protein in Rat Basophilic Leukaemia Cells

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Until now, α2,8-polysialic acid (PSA) chains have been found to modify only neural cell adhesion molecule (NCAM) and the α subunit of the sodium channel in mammalian cells. Using immunofluorescence microscopy and Western immunoblot analysis and an anti-PSA antibody, OL.28, we have found a unique polysialylated protein on the cell surface, in the media, and in an intracellular compartment of the rat basophilic leukaemia cell lines, RBL-1 and RBL-2H3. The intracellular compartment was identified as the Golgi apparatus by colocalization with furin, a TGN protein, and by anterograde distribu-

tion to the ER after treatment with brefeldin A. The polysialylated protein in these cells is not NCAM since northern analysis of RBL poly A+ RNA indicates that these cells do not express NCAM mRNA. In addition, the protein cannot be detected with a series of anti-NCAM monoclonal antibodies or an anti-sodium channel α subunit antibody. Western immunostaining of the 180–260 kDa protein with the OL.28 antibody is abolished by treatment with either *Vibrio cholerae* neuraminidase or PK1A endoneuraminidase. The failure of peptide N-glycosidase F to completely remove the α2,8-PSA structure from the protein, and its sensitivity to β-elimination suggests that the PSA may be found on O-linked oligosaccharides. This polysialylated protein has been purified from RBL-2H3 cell media by ion exchange chromatography on a Q-Sepharose column, followed by affinity chromatography on an OL.28 antibody column. By these methods we hope to isolate the new polysialylated protein, obtain amino acid sequence, and determine its identity.

S22. 11.15am

Molecular Analysis of the Conserved Protein Domains of Sialyltransferases

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The sialyltransferase family consists of a group of enzymes that transfers sialic acid from its nucleotide sugar to various glycolipids and glycoproteins. Cloning of these enzymes and analysis of their protein sequences revealed the presence of two conserved domains present in all the sialyltransferases cloned to date. The larger domain, termed as 'sialylmotif', contains eight invariant amino acids and the smaller one contains two. To address the question on the role of these two domains we have constructed single point mutants by changing the invariant amino acids to alanine using Galβ1,4GlcNAc α2,6-sialyltransferase as a model. In total, 14 such mutants have been constructed that also include conserved amino acids present in these two domains. Each of the mutants was expressed in COS-1 cells, and eight of these retained sialyltransferase activity, allowing comparison of their enzymatic properties with that of the wild type enzyme. Kinetic analysis showed that six out of eight mutants had a 3–12 fold higher K_m for the donor substrate CMP-NeuAc relative to the wild type enzyme, while the K_m values for the acceptor substrate were within 0.5–1.2-fold of the wild type for all eight mutants evaluated. Our results suggest that the larger conserved domain in the sialyltransferase gene family participates in the binding of the common donor substrate, CMP-NeuAc.

S22. 11.30am

Biosynthesis of Polysialic Acid in Mammals and the Regulation of its Expression

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Poly-α-2,8 sialic acid (PSA) is essentially involved in the regulation of mammalian developmental processes and plays an important role as oncodevelopmental antigen in neuroendocrine tumours. The large anionic structure not only modulates NCAM intrinsic homophilic binding functions, but, by sterical means, also influences the interactions of other cell surface molecules. The key enzyme of PSA-synthesis, the eukaryotic polysialyltransferase-1, has recently been identified [1] and demonstrated to catalyse polysialylation of adult NCAM *in vitro*. However, PST-1 did not sialylate any other acceptor molecule tested so far. Having the enzyme available we presently focus on the regulatory mechanisms of PSA-biosynthesis in mammals and evaluation of the dynamics in its expression. FACS analysis with the monoclonal antibody 735 [2] and Northern blot analysis revealed a close correlation between PSA-expression on the cell surface and intensity of the hybridization signal within a broad panel of PSA-positive and PSA-negative cell lines and tissues, suggesting transcriptional regulation. With respect to the question "does alternative splicing regulate NCAM-polysialylation?", a panel of mouse-NCAM-cDNAs which encompass the entire coding region of the three major isoforms but vary in their expression of extra exons π and a were constructed, transfected into a PST-positive human cell line and analysed with the aid of the mouse-NCAM specific mAb H28. Although the two insertions which modify the extracellular part of the NCAM molecule were previously reported to effect NCAM functions reversibly to PSA, under the experimental conditions used in this study all NCAM isoforms were processed, and appeared ectopically as polysialylated molecules.

1. Eckhardt *et al.* (1995) *Nature* **373**: 7150.
2. Frosch *et al.* (1985) *Proc Natl Acad Sci USA* **82**: 1194.

S22. 11.55am

A Possible Polysialylation Signal in the Core Glycan Chain of N-CAM

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Surface expression of polysialic acid (polySia) on animal cells is known to regulate cell-cell interactions and cell migration during fertilization, development, neurogenesis, and tumour metastasis. In spite of the recent success in cloning of $\alpha 2 \rightarrow 8$-polysialyltransferase [1], the molecular mechanism underlying selective expression of polySia on a certain glycoprotein still remains unelucidated. We now present the evidence showing a possible mechanism for N-CAM polysialylation. (a) For polysialylation to occur on its core N-glycan chains it was suggested that at least two distinct sialyltransferases, i.e. CMP-Sia:$\alpha 2 \rightarrow$ 3sialoside $\alpha 2 \rightarrow$ 8-sialyltransferase and CMP-Sia:$\alpha 2 \rightarrow$ 8 sialoside $\alpha 2 \rightarrow$ 8-sialyltransferase, were required for the 'initiation' and 'polymerization' steps. (b) Precise structural analysis of polySia-containing glycan chains isolated from avian and mammalian N-CAM revealed that a major form of the core glycan chains was identified to have an N-linked triantennary structure bearing the novel sulfated GlcNAc residues on the peripheral regions. Such sulfated residues were unique to polysialylated N-CAM molecules and hitherto unreported in other glycoproteins, strongly indicating that sulfation on the

core N-glycan chains may be functioning as a polysialylation signal. (c) A part of the sialic acid residue in the polySia chain was shown to be *O*-acetylated. By analogy with the established KDN-capping on $\alpha 2 \rightarrow 8$-linked polyNeu5Gc chains in rainbow trout polysialoglycoproteins [2, 3] and sulfation at the nonreducing terminal Neu5Gc residues of the Neu5Gc$\alpha 2 \rightarrow$ (5-$O_{glycolyl}$-Neu5Gc$\alpha 2 \rightarrow)_n$ chains in a glycoprotein derived from the egg cell surface complex of sea urchin eggs [4], *O*-acetylation at the nonreducing terminal Neu5Ac residues may possibly be an additional type of termination signal for polysialylation.

1. Eckhardt M *et al.* (1995) *Nature* **373**: 715–18.
2. Kitazume S *et al.* (1994) *J Biol Chem* **269**: 10330–40.
3. Angata T *et al.* (1994) *Glycoconjugate J* **11**: 493–99.
4. Kitazume S *et al.* (1995) this Symposium.

S22 POSTERS

S22

The Eukaryotic CMP-NeuAc: Poly-α-2,8-Sialosyl-Sialyltransferase-1 Represents an Enzyme of High Evolutionary Conservation in Vertebrates

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By expression cloning using a newly generated CHO mutant with the phenotype $NCAM^+/PSA^-$ we were recently successful in isolating the eukaryotic CMP-NeuAc: poly-α-2,8-sialosyl-sialyltransferase-1 (PST-1) from hamster [1] and human origin. Sequencing of the cDNAs revealed identity within the coding regions. Since similarities within the 5′ and 3′ untranslated parts have not been observed, the risk of potential contaminations can be excluded. By PCR a 600 bp fragment from a mouse cDNA-library was amplified and sequenced. Taking the limitations of the PCR approach into consideration, the mouse PST-1 fragment does not show any alterations from the human and hamster sequences. In order to investigate cross hybridization of PST-1 with more distant species, *in situ* hybridization was carried out in parallel on sections of adult and P0 mouse brain, P0 rat cerebellum, and retina of the salamander *Pleurodeles waltl*. The entire coding region of hamster PST-1 was used as a probe. Hybridization signals of equal intensity were obtained in PSA positive tissue sections independent of the species tested. Subsequent immunofluorescence labelling of the same sections with the PSA-specific monoclonal antibody 735 [2] revealed a close correlation between PST-1 and PSA expression. In contrast no sequence homology was found with the *PST* gene of neuroinvasive bacteria [3]. Our results therefore exclude the possibility of a horizontal gene transfer from eukaryotes to eubacteria. However, there is strong evidence for an unexpectedly high evolutionary conservation of PST-1 within vertebrates.

1. Eckhardt *et al.* (1995) *Nature* **373**: 715.
2. Frosch *et al.* (1985) *PNAS* **82**: 1194.
3. Frosch *et al.* (1991) *Mol Microbiol* **5**: 1251.

S22

Cloning, Expression and Site-Directed Mutagenesis of Bacteriophage E Endosialidase

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The endosialidase forming a component of the tail spike of bacteriophage E has been purified and N-terminally sequenced to facilitate cloning of the 76 kDa protein. Sequencing of two clones, which together comprised the deduced endosialidase E open reading frame, revealed a high degree of identity to another bacteriophage endosialidase. Two copies of an 'Asp-box' motif and one copy of a 'P-loop' motif were identified in the deduced amino-acid sequence. The deduced open reading frame encoded a protein of 90 kDa. A full length endosialidase clone has been constructed and translated in a cell-free *E. coli* lysate. The translation product has a molecular weight of 90 kDa and was found to be inactive when assayed for endosialidase activity. The endosialidase gene has been cloned into a prokaryotic expression vector and the enzyme has been expressed as a fusion protein. The purified fusion protein, which is recognized by a polyclonal antiserum raised against non-recombinant 76 kDa protein, has a total molecular weight consistent with the product of the endosialidase gene being 76 kDa. It seems likely that the 90 kDa translation product is post-translationally processed at the C-terminus to give the mature 76 kDa protein.

S22

Interferon-alpha Effect on β-Galactoside α2,6-Sialyltransferase Gene Expression in Human AF10 Myeloma Cell Line

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Interferon-alpha has been used in the treatment of multiple myeloma. We have shown that interferon-alpha 2b (IFN) increases sialyltransferase activity in AF10, a human myeloma cell line that produces IgE. AF10 cell line was incubated for 24 h with or without recombinant IFN at 100–10 000 U ml^{-1}. Sialyltransferase activity was determined by measuring the incorporated radioactivity upon incubation of AF10 cell culture incubation medium supernatant with CMP-[^{14}C]sialic acid in the presence or absence of asialofetuin, an exogenous acceptor of sialic acid. Total cellular RNA was extracted and electrophoresed in 1% agarose formaldehyde gel and transferred to a nylon membrane. ST3, the 1.6 kb cDNA encompassing the complete amino acid coding sequence for β-galactoside α-2,6-sialyltransferase (Gal α2,6-ST). The probe was labelled with [^{32}P]CTP using USB random primer labelling kit. To estimate the total RNA in the samples, the dehybridized membrane was hybridized to T-4 polynucleotide kinase [^{32}P] ATP 5′ end labelled rRNA 18S oligonucleotide probe. In this report we present new evidence to show that the enhanced sialyltransferase activity induced by IFN is associated with an increase in Gal α2,6-ST gene expression in myeloma cells. The elevated level of Gal α2,6-ST mRNA induced by interferon alpha is dose dependent and is correlated with sialyltransferase activity.

S22

Overexpression of the Eukaryotic CMP-NeuAc: Poly-α-2,8-Sialosyl-Sialyltransferase-1 Induces Polysialylation of Unphysiological Acceptor Structures

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α-2,8-Linked polysialic acid (PSA) is a dynamically regulated posttranslational modification of the neural cell adhesion molecule (NCAM). Presence of this large anionic structure profoundly influences NCAM binding properties and has recently been shown to regulate overall cell-cell and cell-substratum interactions. The enzyme catalysing the polymerization of sialic acid, the polysialyltransferase (PST-1), has been cloned [1] and shown to be the only factor necessary for polysialylation of adult NCAM. Northern blot analysis in combination with immunofluorescence staining confirmed a close correlation between PSA-expression and presence of PST-1. With the exception of polysialoglycoproteins in *Salmonidae* and the voltage dependent sodium channel, NCAM seems to be the only structure in vertebrates that can be polysialylated under physiological conditions. However, when PST-1 becomes overexpressed, cells express PSA which is not attached to NCAM. In Western blot analysis with the PSA-specific mAb 735 [2] of cells transiently transfected with PST-1, PSA immunoreactivity is detectable over a broad molecular weight range, superimposing the PSA immunoreactivity between 120 and 250 kDa which is characteristic for polysialylated NCAM. Similar results were obtained with the NCAM-negative cell line LM(tk$^-$). In contrast, a murine cell line which expresses endogenous PST-1, but lacks NCAM, does not contain detectable amounts of PSA before transfection with the physiological acceptor molecule NCAM. The nature of the non-NCAM substrates that are modified by overexpressed PST-1 is currently under investigation.

1. Eckhardt *et al.* (1995) *Nature* **373**: 715.
2. Frosch *et al.* (1985) *Proc Natl Acad Sci USA* **82**: 1194.

S22

In Vivo Incorporation of [^{3}H]Mannosamine into Frog Rhodopsin

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The N-linked oligosaccharide chains of frog (*R. pipiens*) rhodopsin contain sialic acid (NeuAc) [1]. We examined the *in vivo* incorporation of [^{3}H]mannosamine ([^{3}H]ManN) into frog rhodopsin, and its turnover in rod outer segments (ROS). Frogs were injected intravitreally with [^{3}H]Man, either alone or in combination with TRAN[^{35}S]LABEL, and then killed at various post-injection times. Eyes were enucleated and either processed directly for light microscopic autoradiography (LMARG) or initially dissected to obtain neural retinas, from which ROS membranes were isolated. Retinas and ROS were then analysed by SDS-PAGE, ± neuraminidase digestion, either with subsequent fluorography or by excising and solubilizing the band corresponding to opsin and measuring the incorporated radioactivity by liquid scintillation counting. Some ROS samples were treated with 0.2 N H_2SO_4, and the hydrolysates were analysed by HPLC, in comparison with [^{14}C]NeuAc and [^{3}H]ManN. LMARG of eyes injected with [^{3}H]ManN exhibited 'bands' of silver grains at the base of ROS, which became displaced apically with time, consistent with normal ROS membrane protein turnover. At least 85% of the [^{3}H] and ⩾98% of the [^{35}S] in ROS was acid-precipitable and not extractable with $CHCl_3$/MeOH. SDS-PAGE fluorography of ROS showed predominantly one [^{3}H]ManN-labelled band, coincident with opsin; label was absent in the neuraminidase-treated sample. The [^{3}H]/[^{35}S] ratio of ROS and opsin did not change appreciably between 1 day and 3 weeks. ROS acid hydrolysates exhibited comigration of [^{3}H] with [14C]NeuAc, but not with [^{3}H]ManN. These results demonstrate that frog retina can metabolize ManN to NeuAc and incorporate it into rhodopsin, and that the turnover of NeuAc is not independent of that of the polypeptide moiety of rhodopsin.

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S22

A New Assay for the Determination of Sialate 8-*O*-Methyltransferase Activity in Gonads of the Starfish *Asterias rubens*

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Previous work from this laboratory [1–3] demonstrated that the 8-*O*-methylation of sialic acids in starfish takes place at the level of glycoconjugate-bound sialic acids and that it is catalysed by a membrane-bound methyltransferase using *S*-adenosyl-[^{14}C] methionine for transfer. Here we describe a more sensitive and reproducible test using detergent-solubilized enzyme extracts and glutardialdehyde-fixed horse erythrocyte membranes, containing 99% Neu5Gc of total sialic acid. The radioactive, methylated sialic acids bound to the fixed erythrocyte membranes can be separated from the incubation mixture by filtration. Product identification was performed by Radio-TLC with appropriate standards, after release of sialic acids by acid hydrolysis. As a further control for the sialic acid specificity of the methylation, desialylated fixed membranes were tested in the enzyme assay. No radioactive sialic acids could be demonstrated by TLC in the latter case. Using this assay, the impure enzyme was characterized and found to exhibit optimal activity at pH 7.4–8.3. The enzyme can be activated by low concentrations of various divalent metal ions and is inhibited by increasing ionic strength.

1. Schauer R, Wember M (1985) In: *Glycoconjugates*, Proc. VIIth Int. Symposium (E. A. Davidson, J. C. Williams, N. M. Di Ferrate, eds), Vol I (New York: Praeger) pp. 264–65.
2. Bergwerff AA, Hulleman SHD, Kamerling JP, Vliegenthart JFG, Shaw L, Reuter G, Schauer R (1992) *Biochimie* **74**, 25–38.
3. de Freese A, Shaw L, Reuter G, Schauer R (1993) *Glycoconjugate J* **10**: 330.

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S22

Characterization of the Sialic Acids on Recombinant Human Erythropoietin Expressed in Chinese Hamster Ovary Cells

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It has become increasingly apparent that Chinese hamster ovary (CHO) cells can affect modifications, including *N*-glycoylation and *O*-acetylation, to sialic acids of recombinant glycoproteins. Since these substitutions may have biological consequences, we performed an historical survey of the sialic acids present on recombinant human erythropoietin (rHuEpo) produced in CHO cells. The method of Hara *et al.* [1] involving the release of the sialic acids by acid hydrolysis, fluorescent labelling with 1,2-diamino-4,5-methylenedioxybenzene (DMB), and subsequent reverse phase HPLC separation was used. *N*-glycoylation (2.5%) and *O*-acetylation (15% of the total sialic acids) was observed throughout. Treatment of rHuEpo with mild base to remove *O*-acetyl groups had no apparent effect on biological activity in an *in vivo* model. Coupled with the fact that these preparations of rHuEpo have been used successfully in reducing the anaemia observed in patients with chronic renal failure with no adverse indications, these data suggest that *N*-glycoylation and *O*-acetylation of the sialic acids on rHuEpo has no deleterious effect on biological activity.

1. Hara S *et al.* (1989) *Anal Biochem* **179**: 162–66.

S22

α2 → 8-Linked Disialylated Poly-*N*-acetyllactosamine Chains with Le^x and I Antigenic Glycotopes Are Present in the Tetraantennary Arms of a Unique Glycoprotein Isolated from Trout Ovarian Fluid

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Recently, we found the occurrence of a unique glycoprotein in the ovarian fluid of rainbow trout. In this study, we have elucidated the complete structure of a novel type of large tetraantennary *N*-linked glycan chain derived from this glycoprotein. Upon mild acid hydrolysis only Neu5Ac monomer and dimer were released, and the latter was identified as Neu5Ac-α2→8Neu5Ac by ^{1}H-NMR measurements and methylation analysis. The monosialyl and disialyl residues were shown to occur in native glycan chains in an equimolar ratio from data based on mild periodate oxidation. FAB-MS measurement, methylation analysis, and hydrazinolysis-nitrous acid deamination revealed the presence of a poly-*N*-acetyllactosamine skeleton with I antigenic epitope bearing Le^x, sialyl Le^x, and disialyl Le^x [Neu5Acα2 → 8Neu5Acα2 → 3Galβ1 → 4(Fucα1 → 3) GlcNAcβ1→] structures. Oligo/polySia chains larger than the trisialyl group were not detected by FAB-MS determination. The occurrence of disialyl units but not higher oligoSia units on N-linked glycan chains is of particular interest and can be taken as supporting evidence for our recent observation of the involvement of at least two different α2 →8-sialyltransferases in the biosynthesis of α2 →8-polySia chains attached to the core glycans [1].

1. Kitazume S *et al.* (1994) *J Biol Chem* **269**: 10330–40.

S22

Biosynthesis of *N*-Glycolylneuraminic Acid in Echinodermata

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The biosynthesis of *N*-glycolylneuraminic acid (Neu5Gc) in mammals is catalysed by a soluble, cytochrome b_5-dependent monooxygenase which is specific for CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac) [1, 2]. Since the echinodermata are the most primitive organisms known to possess glycoconjugates sialylated with Neu5Gc, the hydroxylase enzyme system in these animals is of considerable interest from an evolutionary point of view. Previous investigations with gonads from the starfish *Asterias rubens* suggested that the CMP-Neu5Ac hydroxylase has very similar cofactor requirements to the enzyme from mammals and that it is also a soluble protein [3]. However, further experiments indicate that the starfish hydroxylase is in fact membrane-associated. Moreover, in stark contrast to the enzyme from mammals, the hydroxylase from *A. rubens* is activated by increased salt concentrations up to 0.4 M with a maximum at 0.1 M NaCl. Experiments with several other salts suggest that the activation is due to increased ionic strength and is not an ion-specific phenomenon. In order to assess to what extent the enzyme from *A. rubens* is typical for the echinodermata, diverse members of this phylum [i.e. starfish (*Ctenodiscus crispatus*), sea urchin (*Stronglyocentrotus pallidus*) and sea cucumber (*Mülleria sp.*)] were studied. In all cases, the hydroxylase was membrane-bound and activated by increased ionic strength. The results demonstrate that the CMP-Neu5Ac hydroxylase in echinodermata differs from the enzyme system in mammals in several important respects.

1. Kawano T, Kozutsumi Y, Kawasaki T, Suzuki A (1994) *J Biol Chem* **269**: 9024–29.
2. Schlenzka W, Shaw L, Schneckenburger P, Schauer R (1994) *Glycobiology* **4**: 675–83.
3. Schlenzka W, Shaw L, Schauer R (1993) *Biochim Biophys Acta* **1161**: 131–38.

S22

Sulfated and Polysialylated Glycosphingolipids Newly Isolated from the Sperm of the Sea Urchin, *Hemicentrotus pulcherrimus*

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Extensive structural diversity in the polySia chains expressed on cell surface glycoproteins has recently been recognized. PolySia chains are expressed not only on *N*- and *O*-glycan chains of glycoproteins but also on glycosphingolipids, although when

expressed on the latter glycoconjugates the degree of polymerization (DP) of the polySia chains thus far reported is low (DP ≤ 3). In polysialoglycoproteins (PSGP) isolated from the eggs of salmonid fish, oligo/polySia chains linked to the *O*-glycan chains are capped by deaminated neuraminic acid (KDN). These KDN-capped oligo/polySia chains are resistant to exosialidases. In this work we isolated a series of mono- and oligosialylglucosylceramides from sea urchin sperm and the following conclusions were drawn. (1) The structures of major gangliosides of *Hemicentrotus pulcherrimus* sperm were determined by chemical and instrumental analyses as: $\pm SO_4^-$ →(8Neu5Acα2→)$_n$→6Glcβ1→1′Cer. (2) Only Neu5Ac was detected as a sialic acid component. (3) OligoSia chains having DP up to 4 were detected though the diSia was predominant. (4) A portion of the oligoSia chains was 8-*O*-sulfated at their nonreducing termini, thereby rendering the chains resistant to exosialidases. (5) Sulfation appeared to terminate the elongation of oligoSia chains. (6) 8-*O*-sulfation at the nonreducing terminal Neu5Ac of disialylated Glc-Cer facilitated inter-residue lactone formation under weakly acidic conditions.

S22

Extended Helical Epitopes of Polysialic Acids

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Vaccine development has been hampered by the poor immunogenicity of α(2-8) polysialic acid capsule of group B *N. meningitidis* which is probably due to structural mimicry with human and animal tissue antigens. Antibodies to α(2-8) polysialic acid can be produced under special circumstances however, all of which recognise an extended helical epitope involving about 10 Neu5Ac units. This is despite the fact that the topological features of the polymer have been shown to be mostly random coil in nature, with the presence of only few extended helical domains. In contrast the *N*-propionylated (NPr) form of α(2-8) polysialic acid is highly immunogenic, and the antibodies, which for the most part do not cross-react with α(2-8) polysialic acid, are bactericidal for group B meningococci. In order to evaluate the specificity of these antibodies a series of mAbs were produced using a tetanus toxoid conjugate of the polymer as vaccine. These antibodies were also predominantly specific for extended helical epitopes consisting of about 10 Neu5Pr units, except that in this case a few were produced which recognised shorter linear sequences $(Neu5Pr)_5$. Of biological interest is the fact that only the former were bactericidal and/or passively protective against group B meningococci. Because group B meningococci do not contain Neu5Pr, it is proposed that the *N*-propionylated form of α(2-8) polysialic acid mimics a compound epitope on the surface of group B meningococci in which the extended helical form of α(2-8) polysialic acid must play an important role.

S22

Carbohydrates from Egg: Their Industrial Scale Preparation and Functionality

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The egg is composed of various important chemical substances that form the basis of life. We have explored the hen egg for industrial scale preparation of a variety of functional carbohydrates. An economical process was established for the large scale preparation of *N*-acetylneuraminic acid (Neu5Ac) from egg. Various oligosaccharides were prepared from glycoproteins of delipidated egg yolk with proteinase. The filtrate from the UF unit of the enzyme hydrolysate was dialysed with a reverse osmosis membrane. The sialyloligosaccharides were purified by the combination of anion exchange and gel filtration chromatography. Their structures were determined by 400 MHz NMR and 2D mapping HPLC and found to be as biantennary complex type sialyloligosaccharides having one or two Neu5Ac and 1-3.5 amino acids.

The absorption and excretion of orally administered labelled egg sialyloligosaccharide was investigated in rats, and 58% of the radioactivity was detected in blood, brain and other organs after 24 h of oral administration. The effect of sialyloligosaccharides on learning performance ability of infant rats was investigated. The group which were orally administered sialyloligosaccharides had a higher success ratio of goal reaching in shorter time as compared to the control group using maze test.

Aliquots of sialyloligosaccharides were added to rotavirus (SA11) and absorbed onto MA-104 cells. The fraction containing 30% sialyloligosaccharides completely inhibited rotavirus infection (IC_{50}; 31.9 mg ml^{-1}) *in vitro*. The inhibition of rotavirus was found at the concentration of 2.5 mg per mouse *in vivo*. A novel compound, Sialylphospholipid, prepared by Neu5Ac and phosphatidylcholine linked through a spacer was 1000-fold more effective as compared to Neu5Ac against a human rotavirus MO strain.

The large scale preparation from egg of the above functional carbohydrates, which are natural and safe, have a potential for application in infant formulations, medical foods and pharmaceuticals.

S22

Characterization of Monoclonal Antibodies against *O*-Acetylated Sialoglycoconjugates and their Application for cDNA Cloning

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The epitope specificities and biological activities of three different monoclonal antibodies (493D4, D1.1, and P-path), all of which recognize *O*-acetylated (Ac) sialic acid-containing glycoconjugates, have been carefully characterized. Only 493D4 was shown to recognize N-linked type sialoglycoproteins in CNS as well as polysialogangliosides such as 9-*O*-Ac-GT3. The antibody, however, had little reactivity with 9-*O*-Ac-GD3. In contrast, P-path reacted solely with ganglioside 9-*O*-Ac-GD3 and 9-*O*-Ac-LD1. D1.1 had binding specificity between the two monoclonal antibodies. Interestingly, P-path had the strongest mitogenic activity on Burkit like lymphoma cells, but not on the

same cell lines possessing Eb virus genome. Using TLC-immunostaining with P-path, P-path-responsive cells were shown to contain 9-*O*-Ac-GD3 as an extremely minor component (0.1 pmol per 10^7 cells). These results suggest the involvement of the ganglioside antigen in signal transduction.

We have cloned a cDNA encoding a factor involved in the formation of *O*-Ac-glycoconjugates by using these monoclonal antibodies as selection probe. Molecular characterization of the cDNA and its product will be presented.

S22

Microheterogeneity of Erythropoietin Carbohydrate Structure: Asn-83 Glycosylation Site

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The microheterogeneity of carbohydrate structures at Asn-83 glycosylation site on recombinant human erythropoietin (rHuEPO) expressed in CHO cells is evaluated by electrospray ionization mass spectrometry of individual glycopeptides. Asn-83 site is known to be occupied by a complex type carbohydrate (tri, tetra-antennary structures and *N*-acetyllactosamine extensions) and the branches typically terminate with neuraminic acids. Conventional carbohydrate analysis uses harsh conditions to cleave the carbohydrate from peptide/protein for separation and identification. Some of the labile groups associated with neuraminic acids can be removed during this process. By using mild conditions for enzymatic digestions and analysing the intact glycopeptides by gentle electrospray ionization we have identified that in addition to the heterogeneity from core carbohydrate branching another level of microheterogeneity exists from differential levels of *O*-acetylation on neuraminic acids. Consistent structural assignments could be made from data obtained using different proteolytic digests, electrospray solvent systems (with acetic or formic acid) and on-line or off-line LC/MS analysis. Using a stepped orifice method designed to specifically identify the glycopeptides, we observe, at higher orifice potential, a series of ions at m/z 292, 334 and 376 (and the related dehydro forms at m/z 274, 316 and 358) corresponding to neuraminic acids with zero, one and two *O*-acetyl groups. The molecular ion region also shows a number of satellite peaks with 42 Da increments. Mild base treatment of glycopeptide with NaOH on ice removed the *O*-acetyl groups without adversely affecting the underlying oligosaccharide structures, resulting in simplified mass spectra.

S22

Molecular Basis for Tissue- and Species-specific Modification of Sialic Acid

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N-Glycolylneuraminic acid (NeuGc) and *N*-acetylneuraminic acid (NeuAc) are two basic members of the sialic acid family. The ratio of NeuGc to NeuAc varies among animal species, and among tissues of a single species. Influenza virus haemagglutinins bind glycoconjugates that contain NeuAc and NeuGc with different affinities, and the diversity of sialic acid may play a role in determining susceptibility to viral infection. A cell adhesion molecule on marginal zone macrophages, sialoadhesin, preferentially recognizes NeuAc-Gal-GalNAc structure but not NeuGc-Gal-GalNAc. These examples suggest that sialic acid difference is biologically important in recognition events mediated by carbohydrates.

We have purified CMP-NeuAc hydroxylase, which forms CMP-NeuGc, from the cytosolic fraction of mouse liver. The amino acid sequences of the purified CMP-NeuAc hydroxylase and peptides obtained on lysylendopeptidase digestion were used to synthesize oligonucleotide primers. A cDNA clone of the enzyme was obtained by the combination of the polymerase chain reaction and rapid amplification of cDNA ends. It was revealed that the enzyme is a unique one whose nucleotide sequence contains no similar sequences in GenBank. Northern blot analysis of various mouse tissues with the enzyme cDNA as a probe suggested that expression of NeuGc is related to the level of CMP-NeuAc hydroxylase mRNA. On Southern blot analysis with the same probe, cross-hybridizing bands were detected in the human genome. Since NeuGc is regarded as one of the tumour-associated antigens, expression analyses of the enzyme in human cells are attracting wide interest.

S22

Sulfated Neu5Gc: A New Type of Capping Signal for Terminating Polysialic Acid (PolySia) Chain Polymerization

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Recently we discovered a new polySia-containing glycoprotein (polySia-gp) in the jelly coat of sea urchin eggs. The structure of the polySia chains was characterized as ($\rightarrow$5-O_{glycolyl}-Neu5Gcα2$\rightarrow$)$_n$ [1]. We now report the identification of a new polySia chain modification in a glycopeptide fraction, designated ESP-Sia, derived from the egg cell surface complex of *Hemicentrotus pulcherrimus*. Most interestingly, a sulfated Neu5Gc residue was found to cap each of the nonreducing termini of the polySia chains in ESP-Sia, thereby forming SO_4^--Neu5Gcα2 $\rightarrow$ (5-O_{glycolyl}-Neu5Gcα2$\rightarrow$)$_n$. The SO_4^--Neu5Gcα2 $\rightarrow$ (5-O_{glycolyl}-Neu5Gcα2$\rightarrow$)$_n$ chains were resistant to *Clostridium perfringens* exo-sialidase, while the uncapped Neu5Gcα2 $\rightarrow$ (5-O_{glycolyl}-Neu5Gcα2$\rightarrow$)$_n$ chains were hydrolysed by this enzyme. The ESP-Sia glycopeptide consisted of 10–14 amino acid residues and contained both N- and O-linked glycan chains. Each O-linked glycan contained a single polySia chain. By analogy with the previously established KDN-capping on polyNeu5Gc chains [2, 3, 4], i.e. KDNα2 $\rightarrow$ (8Neu5-

Gcα2$\rightarrow$)$_n$, sulfation at the nonreducing terminal Neu5Gc residues can be considered as another type of termination signal for *in vivo* polysialylation.

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S22

Selective Ganglioside Desialylation in the Plasma Membrane of Human Neuroblastoma Cells

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A function of the plasma membrane-bound sialidase in the processes of proliferation control and differentiation in human neuroblastoma cells has been suggested [1], but the identity of the sialidase substrate(s) remained unclear. We have therefore now performed a study of the substrate specificity of the enzyme.

Homogenates of human neuroblastoma cells were fractionated on Percoll gradients and the subcellular fractions tested for sialidase activity towards various potential substrates, using very sensitive radiometric assays. Activity towards fetuin and polysialic acid (colominic acid) was found in the lysosomal fractions, but was absent in plasma membranes. On the other hand, plasma membranes contained Triton-activatable sialidase towards the gangliosides GM3, GD1a, GD1b and GT1b, whereas no desialylation of GM1 and GM2 was detectable.

Ganglioside catabolism in intact cells was studied in pulse-chase experiments. For this purpose, gangliosides were metabolically labelled with [3H]galactose and analysed after quantitative isolation and fractionation by HPLC. Addition of the sialidase inhibitor 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid to the culture medium during the chase abolished the plasma membrane-bound sialidase activity but not the lysosomal one, thus allowing discrimination of their functions. The presence of the inhibitor led to a decrease in the catabolism of gangliosides GM3, GD1a, GD1b, GD2, GD3 and GT1b, and the labelling pattern after 7 days of chase remained essentially unchanged. In the absence of the inhibitor, however there was a marked shift during chase from higher gangliosides to GM1, and a strong decrease in GM3.

We conclude that a selective desialylation of gangliosides with terminal sialic acid residues occurs in the plasma membrane of neuroblastoma cells and that the effects of the plasma membrane-bound sialidase on cellular processes are most likely transduced by *in situ* glycolipid modification.

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S22

Sialic Acid, GSLs, and GpIb Contents of Platelet Subpopulations Allow Some New Conclusions as to Their Formation and Destruction

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Blood platelets are a heterogenous population of cells with different densities and volumes. The origin of this heterogeneity is uncertain. It is probably related to the process of thrombopoiesis though some changes in density may occur during the circulating life of platelets. By centrifugation in Percoll density gradients we have separated human platelets into four subpopulations: very light (VL), light (L), intermediate (I) and heavy (H) with densities from 1.022 to 1.08 $g\,ml^{-1}$. Carbohydrate compositions of the subpopulations were largely similar except for VL which exhibited a two-fold reduced content of NANA and a three-fold increased content of GSLs. GSL content of L was similar to that of VL though its NANA content was alike in I and H. By flow cytometry the percentage of cells binding anti-GpIIb-IIIa antibody was close to 100% and similar in all fractions but only about 50% of cells in VL bound antibody to GpIb, the main sialic acid containing glycoprotein of platelets. In some subjects a reduced binding of anti-GpIb was also observed for L but only rarely for I and H. By electron microscopy VL and, to a lesser degree, L exhibited signs of damage. In platelets stored *in vitro* for 48 h a loss of GpIb proceeded at a much faster rate in VL and L than in I and H. The results suggest that the process of destruction of platelets in circulation involves, among others, a loss of GpIb glycoprotein and of sialic acid. High contents of GSLs in VL and L and low GSL contents in I and H may indicate a relation within each pair. Since platelets do not glycosylate ceramides these relations may signify a common origin from a particular class of megakaryocytes.

S22

Production of Monoclonal Antibody to Neolacto Ganglioside with *N*-glycolylneuraminic acid (NeuGc) and Its Expression in Cancer Tissues

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The Hanganutziu-Deicher(HD) antigen is one of the human heterophile antigens, and its antigenic structure was determined to that of *N*-glycolylneuraminic acid (NeuGc)-containing glycosphingolipids (GSLs). Although a number of monoclonal antibodies (MAbs) to gangliosides (NeuAc) have been generated, few MAbs directed to NeuGc-containing gangliosides have been reported. We established three kinds of MAbs against NeuGc-containing gangliosides [1]. One of them, SHS-1, reacted specifically with i-active ganglioside (NeuGc) used as immunogen and not with the structurally related gangliosides such as GM3 (NeuGc) and sialoparagloboside (SPG)(NeuGc), nor other gangliosides (NeuAc). Using the SHS-1 antibody, we investigated the immunohistochemical expression of NeuGc-containing antigen in human colon cancer tissues. Of the cases examined, approximately half reacted with this antibody and normal colorectal tissues gave no reaction. This finding suggests that the expression of NeuGc is enhanced in cancer tissues.

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S22

Two Distinct Sialidases, KDN-Sialidase and NeuAc-Sialidase from the Starfish, *Asterina pectinifera*

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The entrails of the starfish, *Asterina pectinifera*, were found to contain two distinct sialidases. One is a regular sialidase which cleaved sialic acid from sialoglycoconjugates. The other sialidase released KDN from KDN-containing glycoconjugates. Using the 4-methylumbelliferyl α-ketoside of KDN (MU-KDN) and MU-NeuAc as substrates, the KDN-cleaving sialidase (KDN-sialidase) and regular sialidase were purified over 6700-fold and 1300-fold, respectively, from this starfish. The KDN-sialidase was found to have an M_r of 31 000 Da and a pI of 8.3. The regular sialidase, on the other hand, had an M_r of 128 000 and a pI of about 4.8. Hg^{2+} and NeuAc2en were potent inhibitors for the regular sialidase but not for the KDN-sialidase. Using MU-KDN and MU-NeuAc, the pH optima for these sialidases were both determined to be at 4.0. The K_m values for KDN-sialidase and regular sialidase using MU-KDN and MU-NeuAc, respectively, were 0.05 mM and 0.06 mM. The KDN-sialidase hydrolysed MU-KDN 20 times faster than MU-NeuAc, while the regular sialidase hydrolysed MU-NeuAc 88 times faster than MU-KDN. The KDN-sialidase effectively hydrolysed KDN-G_{M3}, KDNα2 $\rightarrow$ 6GalNAc-o1, KDNα2 $\rightarrow$ 6(KDNα2 $\rightarrow$ 3)GalNAc-o1, and KDNα2 $\rightarrow$ 6(GlcNAcβ1 $\rightarrow$ 3)GalNAc-o1. However, under the same conditions, these KDN-containing glycoconjugates were refractory to the regular sialidase. Conversely, G_{M3} is effectively hydrolysed by the regular sialidase but not by the KDN-sialidase. This is the first isolation of a KDN-sialidase from a higher organism that hydrolyses KDN-containing glycoconjugates significantly more efficiently than NeuAc-containing glycoconjugates and also the first report of the coexistence of KDN-sialidase and regular sialidase in the same organism.

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S22

In vitro Synthesis of Poly-α-2,8-*N*-Acetyl Neuraminic Acid: The Job is Done by a Single Enzymatic Activity

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We have recently succeeded in cloning and functional expression of polysialyltransferase-1 (PST-1), the key enzyme of eukaryotic polysialic acid (PSA) synthesis [1]. Availability of this enzyme provides an ideal basis to further analyse its enzymatic properties and to address the central question of: "how many enzymes are involved in conferring PSA to its acceptor molecule NCAM?". In order to facilitate the *in vitro* studies a soluble form of PST-1 was generated by replacing the N-terminal part with a thrombin site and the protein A IgG binding domain [2]. The chimeric protein after expression in COS-M6 cells and affinity purification on IgG-Sepharose was shown to have the expected molecular weight of about 88 kDa. Using a newly developed assay system, which measures the endoneuraminidase NE sensitive incorporation of CMP-^{14}C-NANA into potential acceptor structures, the specificity of PST-1 was assayed with immunopurified embryonic and adult NCAM forms, and with a variety of isolated sugar oligo- and polymers. For additional characterization the resulting products were immunoisolated, before or after treatment with endoneuraminidase NE, separated on SDS-PAGE or high percentage polyacrylamide gels, and identified by autoradiography. None of the isolated sugar molecules was further modified, however, the recombinant PST-1 was efficiently active on both adult and embryonic NCAM. The results presented in our study therefore allow the following conclusions: (i) the polycondensation of PSA in eukaryotes is catalysed by a single enzyme described as PST-1; (ii) the polycondensation of PSA proceeds step by step and does not require preformed oligomeric intermediates; (iii) under the assay conditions used PST-1 is not active on any other molecule than NCAM, indicating that the NCAM primary structure or the N-glycan cores within the 5.Ig-like domain exhibit specific acceptor functions; (iv) under the assay conditions used *in vitro* synthesis of PSA needs PST-1 and the acceptor molecule NCAM. Additional enzymatic activities are not required.

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S22

Characterization of Sialidase Purified from Rabbit Erythrocyte, and Effects of Steroid on Sialidase Activity in Mouse Erythrocyte

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Sialidase activities of rabbit blood cells and serum were measured. Predominant sialidase activity in the blood was detected in erythrocyte particulate fraction when ganglioside was used as substrate. The sialidase for ganglioside was solubilized from the erythrocyte ghosts by using Triton X-100 and purified 1886-fold by sequential chromatographies [1]. The purified enzyme was active towards gangliosides, oligosaccharides and fetuin glycopeptide except for glycoproteins. The active site of this sialidase oriented mainly on the inside of the erythrocyte membrane.

Steroids induce hyperviscosity of blood and hypercoagulability as adverse side effect. Therefore, the relationship between these steroid effects and sialidase activity in erythrocyte membrane was studied in mice. When male C3H/HeN mice (7 weeks old) were injected with 1.6 $mg\,kg^{-1}$ betamethasone phosphate intramuscularly for 7 days, sialidase activity in erythrocyte membrane against ganglioside increased in comparison with that of saline administered control. These results suggest that steroid affects the sialidase activity in mouse erythrocyte membrane. When a kind of Chinese medicine, which has been used for treatment of abnormal blood flow, was administered to the steroid-treated mice orally, the hypercoagulation and sialidase activity in erythrocyte membrane restored to control level.

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S22

Structures of KDN-Containing Sugar Chains Isolated from the Skin Mucus of Loach, *Misgurnus anguillicaudatus*

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After characterizing a deaminated neuraminic acid (3-deoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid, KDN)-containing glycoprotein and purifying its two major sugar chains from the skin mucus of loach, *M. anguillicaudatus* [1], we subsequently isolated four minor sugar chains from the glycoprotein and determined their structures. The sugar chains (oligosaccharide alditols) released by alkaline borohydride treatment were separated into two major sugar chain fractions and a minor sugar chain fraction by Sephadex G-25 (superfine) gel filtration. The structures of the two major sugar chains have been elucidated to be KDNα2 $\rightarrow$ 3(KDNα2 $\rightarrow$ 6)GalNAc-o1 and KDNα2 $\rightarrow$ 6GalNAc-o1 [1]. From the minor fraction, four sugar chains were isolated by HPLC. Using methylation analysis, ^{1}H-NMR spectroscopy, and glycosidase digestion, the structures of the four minor sugar chains were tentatively determined to be NeuAcα2 $\rightarrow$ 6GalNAc-o1, KDNα2 $\rightarrow$ 3(GalNAcβ1 $\rightarrow$ 4)GalNAc-o1, KDNα2 $\rightarrow$ 6(GalNAcα1 $\rightarrow$ 3)GalNAc-o1, and KDNα2 $\rightarrow$ 6(Galβ1 $\rightarrow$ 3)GalNAc-o1. We have proposed a model structure for the KDN-containing glycoprotein from loach skin mucus: the glycoprotein molecule contains 650 KDN-containing sugar chains as described above that are linked to Thr (or Ser) residues and spaced an average of 2.6 amino acid residues apart.

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S22

Expression Cloning of Human Polysialyltransferases

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Polysialic acid is a developmentally regulated carbohydrate composed of α-2,8-linked sialic acid repeats. By expression cloning using COS-1 cells, we have cloned human enzymes responsible for polysialylation in glycoproteins (PST) and glycolipids (GT3ST). In order to clone these enzymes, COS-1 cells were co-transfected with NCAM cDNA and a human brain cDNA library (for PST) or GD3ST cDNA and a human melanoma MEL-28 cDNA library (for GT3ST). Transfected cells were enriched with 735 or M6703 antibody by cell sorting. Siblings selection of enriched plasmids in separate experiments resulted in two clones termed PST and GT3ST.

The cloned PST shares a sialyl motif with the sialyltransferase gene family but with the distinction of basic amino acid clusters. PST forms polysialic acid on NCAM judged by Western blotting. The tissue distribution of PST mRNA correlates well with the presence of polysialic acid detected by antibody 735 in various foetal and adult human tissues. Especially in the brain, PST mRNA is expressed much more in the foetus than in the adult.

Surprisingly, GT3ST was found to be identical to GD3ST. The results obtained indicate that GT3 is formed when GD3/GT3ST is present in large quantity. Work is in progress to determine if GT3ST also forms a tetrasialosyl linkage. These newly cloned enzymes will be powerful tools in understanding the roles of polysialylation in glycoproteins and glycolipids.

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S22

Suppression of Cell-proliferation by DNA-Alginic Acid Complex

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DNA, one of the most important materials in the life process, can be regarded as a naturally occurring, highly specific functional biopolymer. In this study, the effect of DNA on the non-specific suppression of cell proliferation was investigated *in vitro*. The native high molecular weight DNA showed a larger effect on the suppression of cell-proliferation than the single strand low molecular weight DNA. However, single strand high molecular weight DNA had a more potent effect than the double strand native DNA. Direct interaction of the native DNA with cells was confirmed by fluorescence microscope. It was also shown that the native DNA was more effective in fibroblasts than in epidermal cells.

It is well known that negatively charged polysaccharides suppress the proliferation of cells. Alginic acid is an unbranched polysaccharide contained in sea plants and microorganisms. It forms a specific water-insoluble 'egg-box' structure by coordination with cationic metals such as calcium ions.

Because the native DNA is water-soluble and not stable after removal from the nucleus, its usefulness as a functional biopolymer is quite limited. To suppress the proliferation of cells effectively, blended substrates composed of DNA and alginic acid were prepared. When DNA was immobilized by alginic acid and used for cell culture, suppression of cell-proliferation increased. Since the effect of DNA or alginic acid alone was very small, the effective suppression would be caused by potentiation by the DNA-alginic acid complex. Consequently, by selecting a suitable DNA-alginic complex, optional suppression could be expected.

S22

Changes In Sialidase Activities and in Cell Surface Sialylation in U-937 Cells Undergoing Apoptosis

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Dramatic changes in sialidase activities are known to occur

during major phenotype changes such as cell differentiation or activation and there is evidence to suggest that these enzymes might modulate certain cellular functions and intercellular signalling by influencing cell surface sialylation. We, therefore, investigated whether alterations of this enzymatic system might be associated with apoptosis, a programmed form of cell death accompanied by complex morphological and biochemical changes which, in many cases, remains to be fully elucidated.

Sialidase activities toward various natural and synthetic substrates were determined in myelomonocytic cells U-937 at various culture times in the same medium (1–12 days). In these conditions, U-937 showed consistent signs of apoptosis after 5 days of culture as revealed by standard cytological techniques, cell death detection ELISA and DNA fragmentation pattern. The levels of sialidase activity toward all tested substrates were found to change reproducibly as a function of culture time, with each substrate exhibiting a specific fluctuation pattern. In parallel, the extent of cell-surface sialylation decreased regularly in a time-dependent fashion.

Cell surface hyposialylation is believed to favour the recognition and the elimination of apoptotic cells by phagocytes. Therfore, experiments are currently in progress to determine whether the changes in sialidase activities observed here are involved in the reduced cell surface sialylation of apoptotic U-937.

Supported in part by EU grant EV5V-CT94-O399.

S22

lec 32: A New Mutation in CHO Cells that Abrogates CMP-Sialic Acid Synthetase Activity

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LEC29.Lec32, a cell line isolated in screens of MNNG-treated populations of CHO cells for rare mutants that produce $\alpha(1,3)$fucosyltransferase activities, exhibits an unusually high sensitivity (about 300×) to the toxic lectin ricin. This is often indicative of an increased exposure of galactose residues on cell surface glycopeptides. Analysis of LEC29.Lec32 cell-surface glycoproteins by both lectin affinity chromatography and HPAE-PAD have shown a nearly complete lack of sialic acid residues. Genetic analysis employing cell hybridization techniques demonstrated that the *lec32* mutation is recessive and novel since hybrids formed with Lec2 or Lec3 sialic acid-deficient CHO mutants exhibited complementation.

Cell-free extracts of LEC29.Lec32 cells were found to have normal levels of a sialyltransferase which recognizes asialofetuin as substrate. However, CMP-sialic acid (CMP-SA) synthetase activity was completely absent in LEC29.Lec32 extracts when measured by a sensitive radiochemical assay procedure. As expected, the mutant cells contain less than 5% of CMP-SA compared to parental CHO cells which possess about 1.6 nmol per mg cell protein. The properties of the CMP-SA synthetase in parental CHO cells appear to be essentially identical to those reported for the same activity in various mammalian tissues with regards to pH optimum, cation requirements, sensitivity to various inhibitors and K_m values for both CTP and SA. Revertants isolated by selection in low concentrations of ricin have regained normal cell levels of enzyme activity.

S22

Microheterogeneity of Erythropoietin Carbohydrate Structure: N-24, N-38 and O-126 Glycosylation Sites

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Site specific microheterogeneity of the carbohydrate structures on glycosylation sites N-24, N-38 and O-126 of recombinant human erythropoietin (rHuEPO) expressed in CHO cells has been evaluated by electrospray ionization (ESI) mass spectrometry. The microheterogeneity is largely associated with the presence or absence of terminal *N*-acetylneuraminic acid residues (Neu5Ac), to varying amounts of *O*-acetylation of Neu5Ac residues and, to the presence or absence of *N*-acetyllactosamine extensions. All rHuEPO glycosylation sites exhibited some level of *O*-acetylation of Neu5Ac residues, but N-24 and N-38 appeared to exhibit mainly mono-*O*-acetyl derivatization. Site specific differences in *O*-acetyl-Neu5Ac may be due to sample handling of labile structures. The O-126 site was a simple mucin type structure; MS/MS analysis confirms the assigned mucin structure with one or two Neu5Ac residues, respectively. Consistent structural assignments could be made from data obtained using ESI solvent systems (aqueous/methanol with acetic or formic acids), on-line or off-line LC/MS analysis. Complex type carbohydrate branching patterns were observed that were consistent with known asialo branching structures. All site specific glycopeptides were isolated from reverse phase column chromatography of endoproteinase Glu-C digestions of nonreduced rHuEPO.

S22

Characterization of Antigenic Specificity of Different Anti-($\alpha 2 \rightarrow 8$-Linked Polysialic Acid) Antibodies: Anti-polyNeu5Ac, Anti-polyNeu5Gc, and Anti-polyKDN Antibodies

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Polysialic acids (PSAs) represent a group of polymers of *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), and deaminated neuraminic acid (KDN). PSA is recognized as an oncodevelopmental antigen and known to function as a regulator of cell adhesion and cell migration. In elucidating the structural diversity and functional roles of PSAs using anti-PSA antibodies, it is essential to define the immunospecificities of the individual anti-PSA antibodies, such as dependence of their immunoreactivities on PSA chain length. In this study we have developed a rapid and sensitive method for immunodetection of oligo/polySia epitopes in ELISA analysis using oligo/polySia chains conjugated with the phosphatidylethanolamine dipalmitoyl group. Use of a series of such lipidated oligoSia with the known DP allowed us to determine the precise chain lengths required for the binding of different

antibodies. We report the results of such studies for five different antibodies including anti-polyNeu5Ac antibodies (H.46, 12E3, and 5A5), a newly developed anti-polyNeu5Gc antibody (2-4B), and anti-polyKDN antibody (kdn8kdn).

S22

Study of 7(9)-*O*-Acetylation of Sialic Acids in Bovine Submandibular Glands

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O-Acetylation of sialic acids was for a long time considered as an uncommon modification found on certain salivary mucins and neural gangliosides. It has been demonstrated during recent years that this chemical reaction is a frequent event in some species of microorganisms and higher animals including man. This reaction is regulated in a molecule-specific, cell-specific, tissue-specific, development-specific and tumour-specific fashion. Furthermore, the importance of *O*-acetylated sialic acids has recently been strengthened, since it has been shown that they play different primordial biological roles and are also involved in human pathology such as infection and cancer. It seems that at least two distinct enzymes are responsible for *O*-acetylation; the acetyl-CoA: *N*-acylneuraminate 4-*O*-acetyltransferase (EC 2.3.1.44) and the acetyl-CoA: *N*-acylneuraminate 7(9)-*O*-acetyltransferase (EC 2.3.1.45) [1]. So far, only the 7(9)-*O*-acetyltransferase activity of rat liver has successfully been studied [2–4]. Investigation of this activity in bovine submandibular glands, a system in which the 7(9)-*O*-acetyltransferase was described for the first time, allowed us to determine the subcellular localization of the enzyme, and to characterize its activity, acceptor specificity and other enzymatic properties, as well as the nature of the neo-acetylated sialic acid residues.

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S22

Characterization and Purification of the α2,8-Polysialyltransferase from Embryonic Chick Brain

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The CMP-Neu5Ac(Sia): α2,8-polysialyltransferase (polyST) from embryonic chick brain has been studied using the established polyST assay [1]. This Golgi enzyme catalyses synthesis of the α2,8-linked polysialic acid (polySia) chains that cap N-linked oligosaccharides on neural cell adhesion molecules (NCAM). The α2,8-linked polySia glycotope is expressed in organisms as diverse as neuroinvasive *Escherichia coli* K1, the eggs of certain fish species, the nerve tissue of vertebrates, and on a number of human tumours. In the chick embryo brain, optimal polyST activity was expressed at 14 days and in the presence of 1 mM DTT and 10 mM $MnCl_2$. $MnCl_2$ (10 mM) increased polyST activity ~five-fold. Kinetic studies showed the K_m for CMP-Neu5Ac to be ~133 μM at 33 °C, pH 6.1. Partial purification of the chick brain polyST was facilitated after detergent solubilization using CHAPS or sodium cholate and affinity column chromatography. The detergent-protein ratio was critical for retaining activity after solubilization. Heparin was shown to be a competitive inhibitor of the polyST. While it is important during solubilization/purification to optimize polyST activity, it is also useful to find ways to decrease or inhibit the activities of the other (e.g. α2,3-;α2,6-) sialyltransferases. To this end, temperature differential studies have shown that it is possible to selectively lower the other sialyltransferase activities without significantly affecting polyST activity. In addition, increasing concentrations of colominic acid appear to inhibit the activities of the other sialyltransferases and increase polyST activity. Polysialylation of the endogenous NCAM acceptor appears to be by a processive mechanism, which is similar to how the polySia chains in E. coli K1 [2] and trout egg polysialoglycoproteins are elongated after chain initiation [3].

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S22

Purification of a Potential Initiating α-2,8-Sialyltransferase for the Biosynthesis of Polysialic Acid from Human Neuroblastoma Cells

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The biosynthesis of polysialic acid provides important developmental regulation, however the steps necessary for the synthesis of this large polymer in humans are not clearly defined [1]. It is proposed that an initiating enzyme is required to transfer the first NeuAc(α2,8) to an existing NeuAc(α2,3) and the extension of the polymer should have at least one additional enzyme. Extracts of human neuroblastoma, CHP-134 cells, synthesized polymers of polysialic acid of DP-10 or longer as demonstrated by the binding to an immunoaffinity column which detects polysialic acid [2]. Upon further fractionation of the extract and subsequent purification on PAZ-agarose-colominic acid [2] the ability to synthesize the polymer was lost. However, a highly purified enzyme which was recovered after these procedures retained the ability to transfer [^{14}C]NeuAc from CMP-[^{14}C]NeuAc to sialylated fetuin but not asialofetuin. The substrate specificity reflects a characteristic necessary for an α-2,8-sialyltransferase as an initiating enzyme for the biosynthesis of polysialic acid. K_m values and other properties of the purified enzyme will be given. An initiating enzyme was reported recently in trout eggs [3], however evidence for the enzyme was not observed by others [4].

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S22

Expression of CMP-*N*-Acetylneuraminic Acid Hydroxylase in COS-1 Cells through Transfection of cDNA Clones

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CMP-*N*-acetylneuraminic acid (CMP-NeuAc) hydroxylase, which converts CMP-NeuAc to CMP-*N*-glycolylneuraminic acid (CMP-NeuGc), is a key enzyme for the production of NeuGc-containing glycoconjugates. The CMP-NeuAc hydroxylation step requires an electron transport system of several components including cytochrome b_5, which transfers electrons from NAD(P)H to CMP-NeuAc hydroxylase. Recently, cDNA encoding CMP-NeuAc hydroxylase was isolated [1]. Analysis with RT-PCR revealed the presence of two types of mRNAs. Type 1 has an open reading frame with 1734 bp and type 2 is a truncated form with a 138 bp-deletion without frameshift. In order to study the function of these mRNAs, two types of cDNAs were transfected into COS-1 cells. Immunofluorescent staining revealed that the protein encoded by type 1 mRNA localized in the cytosol fraction and that from type 2 in the perinuclear area. The NeuGc content was increased in the COS-1 cells transfected with type 1 cDNA but not in the cells with type 2 cDNA, suggesting that only the type 1 mRNA encodes the active CMP-NeuAc hydroxylase. With regard to the fact that the cytosol fraction lacks the soluble form cytochrome b_5 in COS-1 cells, these results suggest that the membrane-bound cytochrome b_5 is involved in the CMP-NeuAc hydroxylation in the transfected COS-1 cells.

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S22

Characterization of Sialidase from the Ovary of Starfish, *Asterina pectinifera*

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Sialidase is widely distributed in living organisms and participates in the metabolism of sialoglycoconjugates. During work on the isolation of sialidases, we found that high sialidase activity exists in the ovary of the starfish, *Asterina pectinifera*, but not in the seminal gland. A sialidase has been purified from the ovary of starfish by means of procedures comprising extraction, ammonium sulfate precipitation and chromatographies on Octyl-Sepharose, CM-Sephadex, Chelate Cellulofine and Sephadex G-100. The final preparation was free from β-galactosidase and other glycosidase activities. The enzyme was most active at pH 4.0 for artifical substrate and pH 3.5–4.0 for natural substrates, and was stable at pH 4–5. The enzyme liberated the sialic acid residues from (α2–3) and (α2–6) sialyllactose, colomic acid, fetuin peptide, and transferrin, but not bovine submaxillary mucin. The enzyme also hydrolysed gangliosides GM_3 and GD_{1a} in the presence of sodium cholate as a detergent.

S22

Role of Arginine in *Escherichia coli* K1 CMP-*N*-Acetylneuraminic Acid Synthetase

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CMP-*N*-acetylneuraminic acid synthetase catalyses the synthesis of the substrate for all known sialyltransferases. The enzyme catalyses the reaction CTP + β-NeuNAc $\rightarrow$ CMP-β-NeuNAc + PPi. The CMP-NeuNAc synthetase of two polysialic acid producing bacteria, *Escherichia coli* K1 and *Neisseria meningitidis* group B, have been sequenced and purified to homogeneity. In *E. coli* K1 this enzyme is encoded by the *neuA* gene. We have investigated the role of amino acid residues in the active site of the *E. coli* K1 enzyme by chemical modification and site directed mutagenesis.

E. coli K1 CMP-NeuNAc synthetase activity is sensitive to the arginine modification reagent phenylglyoxal. Alignment of the deduced amino acid sequences of CMP-NeuNAc synthetase from *E. coli* K1, *N. meningitidis*, and Group B Streptococcus revealed a consensus sequence in the amino terminus containing a conserved arginine residue at position 12. To examine the role of arg-12 in catalysis, mutations in the *neuA* gene of *E. coli* K1 were constructed. Site directed mutation of this arginine to glycine or alanine resulted in no detectable enzyme activity in cell lysates, although enzyme produced in these mutants could be detected by immunoblots. In an experiment in which R12 was randomly mutated only Arg revertants were detected as positive in colony assays. On the other hand site specific mutation of Arg to Lys yields an active enzyme. The R12K mutant was purified to homogeneity and characterized. The R12K mutant has a two-to-three-fold higher K_m for NeuNAc than the wild type enzyme. Other properties of this enzyme such as pH optimum, CTP K_m are very similar to the wild type. The results suggest that a positively charged residue may be important at position 12.

S22

Changes in Sialidase Activities During *In Vivo* Ageing of Human Erythrocytes

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Human erythrocytes appear to possess plasma membrane bound sialidase, linked to the membrane, at least in part, by a glycosylphosphatidylinositol anchor (GPI), and located on the external surface of the membrane. The present work describes the diversity of sialidase pattern in young and old erythrocyte membranes.

Red cell separation, in a discontinuous Percoll density gradient, allowed young or old cell-fraction enrichment; the young and old erythrocytes membranes were prepared and sialidase activity was determined. In young ghosts, sialidase activity on MU-NeuAc was 1.5 times as much as the oldest (23 μU mg^{-1} young ghost protein compared to 15.1 μU mg^{-1} old ghost protein), the optimal pH for both sialidases was 4.2. The sialidase carried in human young and old ghosts can be released by treatment with PIPLC from *Bacillus cereus*, indicating that

the enzyme, in both young and old ghosts, is linked to the membrane by a GPI. The maximal release obtained is about 71.7% for young ghosts and 33.4% for the oldest. The fact that the release of old ghost sialidase appears to be 50% lower compared to young ghost sialidase can be interpreted as: a greater resistance to the action of PIPLC; or a decreased amount of old ghost sialidase anchored to the membrane by GPI.

S23. CARBOHYDRATES AND CELL ADHESION I: SIALOADHESIN, CD44, CD22 AND GLYCOSYLATION AFFECTING INTEGRINS AND OTHER ADHESION MOLECULES

Chairs: Ajit Varki, Ricardo Brentani

S23. 9.50am

MEL-85 LBM, a Sulfated and β_1-Integrin Associated Molecule, as a Possible Laminin Co-receptor

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Using an LN overlay assay it was possible to identify a laminin binding molecule (LBM) in Mel-85 cells, a human melanoma cell line. This molecule, Mel-85 LBM, has an apparent molecular mass of 100–110 kDa, a negative net charge at physiological pH (pI = 5.9) and is metabolically radiolabelled with sodium sulfate (^{35}S). Data suggest that this sulfated molecule is physically associated with integrin, since it could be co-purified together with $\alpha_7\beta_1$, a laminin binding integrin, by laminin affinity chromatography and it could be co-immunoprecipitated using an antibody specific for β_1 integrin. In spite of this, the ability of Mel-85 LBM to bind laminin is independent of integrin association since even after electrophoretic separation, Mel-85 LBM binds laminin in overlay assays. Mel-85 LBM was shown to be present at the cell surface, as it was iodinated by ^{125}I/lactoperoxidase, and to be a tightly membrane-bound molecule since it was extractable by a TX-100 containing buffer but not by a saline/EDTA buffer. We are proposing that this sulfated molecule could act as a laminin co-receptor mediating laminin-integrin interactions and that it may have a crucial role for the biological activity of laminin.

LBM binds laminin in a divalent cation-dependent way and chemical reduction with β-mercaptoethanol decreases its molecular weight to 95–100 kDa, but does not affect its ability to bind laminin. Since Mel-85 LBM is eluted from a WGA-Sepharose column and has its molecular weight reduced after N- or O-glycosidase treatment, we concluded that there are carbohydrate side chains (N- or O-linked) in this molecule, and moreover that such side chains are not important to the LBM-laminin interaction, because N- or O-glycosidase treatment does not abolish it. Interestingly, however, chemical oxidation with increasingly concentrated solutions of sodium metaperiodate completely inhibited this ability, evidencing that other sugar residues may play a role in its laminin binding activity. This last result and the ability of soluble heparin to completely inhibit the laminin binding activity of Mel-85 LBM suggest that this sulfated molecule may have glycosaminoglycan chains and that LBM could have a proteoglycan nature.

S23. 10.10am

Involvement of Syndecan-2 in Cytoskeletal Organization

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On implantation of mouse lung and colon carcinoma-derived clones with different metastatic potentials into various tissues of mice, low metastatic clones with the capacity to elicit the host stromal response exhibited tumourigenesis depending on the interstitial matrix formed by the induced stromal cells. On the contrary, growth of highly metastatic clones that elicited no significant stromal response and formed a well-organized basement membrane (tumour basement membrane), depended on the tumour basement membrane *in vivo*.

In vitro analyses of adhesive responses of these clones to fibronectin, a major constituent of the induced matrix, and also to recombinant fibronectin polypeptides containing RGD cell-binding domain, C-terminal heparin-binding domain or these two domains in a fused form, clearly demonstrated that the low metastatic cells formed stress fibres under the co-operation of integrin $\alpha5\beta1$ and syndecan-2. On the other hand, in the highly metastatic cells expressing the integrin at the same level as the low metastatic cells but expressing a lower level (1/6) of syndecan-2, the pericellular localization of actin fibrils was induced, resulting in the formation of a ruffling membrane. These results indicate that the level of expression of syndecan-2 is of critical importance for the manner of the cytoskeletal organization.

Moreover, we demonstrated that syndecan-2 exhibited a specific binding to fibronectin *via* its heparan sulfate chains having a structure of $[IdoA(2OSO_3^-)\text{-}GlcNSO_3^-(6OSO_3^-)]_5$. In addition, syndecan-2 was shown to be phosphorylated at only serine residue(s) in a cytoplasmic domain of the core protein, suggesting that phosphorylation/dephosphorylation, probably occurring at this site, is the earliest event induced intracellularly by its binding extracellularly to fibronectin.

S23. 10.30am

Interaction of XB Cadherins with Human Blood Type B Active Determinants in Frog Embryonic Cells

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We previously reported the occurrence of novel fucose-containing pentaglycosylceramide with blood type B active determinant in *Xenopus* blastula cells [1]. A monoclonal antibody raised against this novel ceramide completely disrupted Ca^{2+} dependent cell-cell adhesion of frog blastula cells. Several commercially available monoclonal antibodies against human blood B determinant also disrupted Ca^{2+} dependent adhesion of frog cells. The blood group B active determinants were detected on several glycolipids and glycoproteins. The blood group B active determinants were distributed in cell-cell contact areas and in membrane systems of frog embryonic cells.

We previously showed that Ca^{2+} dependent cell-cell adhesion of early embryonic cells is mediated by cadherin systems [2]. Since the monoclonal antibodies against blood group B active determinant disrupted Ca^{2+} dependent cell-cell adhesion of frog embryonic cells completely, we examined whether cadherins and blood group B antigens are interacting with each other. For this purpose, we raised a monoclonal antibody against frog XB cadherin which disrupts Ca^{2+} dependent cell-cell adhesion of frog embryonic cells. Using a laser scanning confocal microscope, the colocalization of these two antigens was clearly demonstrated and the possible formation of a supramolecular complex consisting of blood group B active molecules and cadherins was shown by a resonance energy transfer method using monoclonal antibodies against these two molecules.

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S23. 10.50am

I-Type Lectins. Carbohydrate-binding Proteins of the Immunoglobulin Superfamily

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Mammalian lectins are classified based on protein sequence homologies. A new lectin family has emerged from independent investigations of CD22, sialoadhesin (Sn), myelin-associated glycoprotein (MAG) and CD33. Unlike all previously known animal lectins, these belong to the immunoglobulin superfamily, suggesting the name 'I-type' lectins. Other possible members include the neural cell-adhesion molecule and the intercellular adhesion molecule-1. All are integral membrane proteins, and some have large cytosolic domains with phosphorylation sites. This talk will focus primarily on the lectin properties of CD22, which involve recognition of the motif Siaα2-6Galβ1-4GlcNAcβ1- (other α2-6 linked Sia structures e.g. linkage to GalNAc and GlcNAc are also recognized with a lower affinity). In contrast, Sn, MAG and CD33 bind various Siaα2-3 containing structures. Potential ligands may be 'masked' by 9-*O*-acetylation of Sias. When CD22 is coexpressed with α2-6 sialyltransferase, the lectin property is lost, but can be restored by sialidases. Similar abrogation of lectin function *in cis* by endogenous ligands has been reported for CD33 and MAG. Certain sialoglycoproteins are superior ligands for CD22, and presumably interact in a multivalent fashion with cell surface CD22 which is itself presented in a multimeric form. All these lectins must function in a milieu of natural biological fluids containing high concentrations of sialylated glycoproteins. Thus, while IgM and Haptoglobin are the only high affinity ligands for CD22 in plasma, many other sialoglycoproteins also interact, with a lower affinity. However, IgM is present at much lower concentrations in the extracellular fluid. Thus, functioning of these lectins may be triggered only when the soluble inhibitor concentration falls below a threshold level in certain tissue compartments.

S23. 11.10am

The First Immunoglobulin-Like Domain of Sialoadhesin Contains the Sialic Acid Binding Site: A Comparison with CD22

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Sialoadhesin (Sn) and CD22 are members of a novel family of sialic acid-dependent adhesion molecules belonging to the immunoglobulin superfamily. Sn is a macrophage restricted receptor with 17 extracellular Ig-like domains recognizing NeuAcα2,3Gal whereas CD22 is a B cell restricted receptor with 7 Ig-like domains that recognizes NeuAcα2,6Gal. Sequence similarity between these proteins is highest within their first four N-terminal Ig-like domains. Here we localize the binding site of both molecules by generating a series of extracellular domain deletion mutants fused to the Fc portion of human IgG1. Binding capacity was analysed both by solid phase cell adhesion assays and also by surface plasmon resonance using purified glycophorin and CD45 as ligands for Sn and CD22 respectively. For Sn, construct domain 1 was both necessary and sufficient to mediate sialic acid dependent adhesion of the correct specificity. In contrast, for CD22, both domains 1 and 2 together were required for sialic acid dependent binding.

S23. 11.30am

Phosphorylation of the Neural Cell Adhesion Molecule L1 is Involved in Oligomannosidic Glycan Mediated Interaction with NCAM and Neurite Outgrowth

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Some members of the immunoglobulin(Ig) superfamily can mediate cell recognition and adhesion by protein-protein and by carbohydrate-protein interactions [1]. We have previously shown that two members of the Ig superfamily, the neural cell adhesion molecules L1 and NCAM, associate through oligomannosidic glycans carried by L1 thus implying NCAM to function as a carbohydrate binding protein. Extending earlier studies on the functional role of the L1/NCAM association on neurite outgrowth of cerebellar neurons in culture [2], we investigated signal transduction mechanisms elicited by this

association. We present evidence that concomitant with reduced neurite outgrowth in the presence of inhibitors of the L1/NCAM interaction, phosphorylation of serine and possibly also tyrosine residues of L1 itself is affected. This observation suggests phosphorylation of L1 to be one step in the signalling cascade implicated in neurite outgrowth.

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S23. 11.50am

Carbohydrate-Carbohydrate Binding Strength Related to Cell Adhesion Measured by Atomic Force Microscopy

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Cell adhesion is a cascade of multistep processes involving homophilic and heterophilic protein-protein, protein-carbohydrate and carbohydrate-carbohydrate interactions. Measurements of binding strength intrinsic to cell adhesion molecules is necessary to assess their contribution to the maintenance of the anatomical integrity of multicellular organisms. Until now these forces for any type of intermolecular interactions have remained unknown. Atomic force microscopy was used to measure directly the binding strength between cell adhesion proteoglycans from a marine sponge. Under physiological conditions the adhesive carbohydrate-carbohydrate binding force between two cell adhesion proteoglycans was found to be up to 400 pN [1]. Thus a single pair of molecules could hold the weight of 1600 cells. High intermolecular carbohydrate-carbohydrate binding forces are likely to form an important basis for the integrity of the multicellular sponge organism. These results provide the first and essential evidence that carbohydrate-carbohydrate interactions can perform the cell adhesion function that we have assigned to it.

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S23 POSTERS

S23

Mouse Sperm-Egg Binding: Enzyme-Assisted Synthesis of Potential Carbohydrate Ligands

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Carbohydrate-protein interactions play an important role in various intercellular recognition events. In the case of mouse sperm-egg binding, the initial species-specific interaction is partly mediated by the oligosaccharides of the zona pellucida glycoprotein-3 (ZP3). Terminal α1,3-linked galactose residues on the O-linked carbohydrate chains of ZP3 have been shown to be required for binding [1] but a role has also been proposed for sperm cell-surface β1,4-galactosyltransferase [2].

One strategy to investigate the role of carbohydrates in the murine sperm-oocyte interaction is to test an array of structurally diverse oligosaccharides as competitive inhibitors. Therefore, we have produced by enzyme-assisted synthesis, using both natural and recombinant glycosyltransferases, a defined series of *N*-acetyllactosamine oligomers which are linked to a synthetic spacer molecule SP (SP = $(CH_2)_8COOCH_3$ or $(CH_2)_3NH_2$). The oligo(*N*-acetyllactosamine) backbones have been substituted with Fuc, NeuAc and α1,3-linked Gal residues to produce the desired oligosaccharide variants. Multi-step reactions were performed by combining two or more glycosyltransferases in the incubation mixtures. Reactions involving β1,4- and α1,3-galactosyltransferases were carried out using *in situ* generation of UDP-Gal. The purified products were characterized by ^{1}H-NMR spectroscopy.

The compounds thus produced will be tested in mono- and/or multivalent form for their ability to inhibit mouse-sperm egg binding *in vitro*.

This research was financially supported by Human Frontier Science Research Grant No 414-94M.

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S23

Disruption of the Mouse Basigin Gene, which Encodes a Carrier of Le^x Epitopes in Embryonal Carcinoma Cells

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Basigin was found as a carrier of Le^x antigen in embryonal carcinoma cells and belongs to the immunoglobulin superfamily [1]. The protein portion of basigin is 30 kDa, while the glycosylated molecule is 43–66 kDa. The Ig-like domain of basigin is unique, since it has a strong homology to both the β-chain of major histocompatibility class II antigen and the Ig V domain. Basigin is expressed in various embryonic and adult cells, while in the brain it is recognized as a blood-brain barrier antigen HT7. Basigin is also identical to gP42, which was found to be co-purified with integrin.

We have found that transfection with fucosyltransferase IV (α-1,3-fucosyltransferase) cDNA into L cells enhances integrin-mediated cell-substratum adhesion [2]. One of the newly fucosylated molecules in the transfected L cells was identified as basigin, which co-precipitates with integrin upon immunoprecipitation. Integrin was not newly fucosylated. Thus, we have proposed that fucosylation of basigin enhances integrin-mediated cell-substratum adhesion.

In order to gain direct evidence for the function of basigin, we tried to generate mice homozygous for the disruption of the basigin gene. Mouse basigin gene (*bsg*) is on Chr19, and spans 13.5 kb, and the coding region is distributed over 7 exons. We isolated *bsg* from BALB/C mice and determined the whole structure. The targeting construct was designed to delete the 1st exon by a positive and negative method using a neomycin-resistance gene and diphtheria toxin gene. We transfected the construct to D3 ES cells, selected the homologously recombined

ES cells, and injected them into blastocysts. The apparently homozygous mice with the disrupted gene so far generated did not show distinct abnormalities. Experiments are in progress to disclose altered phenotype in basigin-negative mice and in cells derived from them.

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S23

Ganglioside GD1α as an Adhesion Molecule of Murine Metastatic Tumour Cells

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Glycosphingolipid composition of a murine lymphosarcoma cell line RAW117-P and its subline H10 which exhibits high metastatic potential to the liver have been studied. Both cell lines contained LacCer, Gg_3Cer, Gg_4Cer, GM1b and GD1α. The content of GD1α in H10 was much higher than that in the parental cells. GD1α was found to inhibit the adhesion of H10 to the hepatic sinusoidal endothelial cells (HSE). The HSE were found to adhere and grow preferentially on the area coated with GD1α but not on those coated with other glycosphingolipids. Proteins having an affinity for GD1α were isolated by binding experiments of ^{35}S labelled membrane proteins of HSE. Immunoblot of plasma membrane proteins of H10 with anti-GD1α antibody showed the presence of glycoproteins containing GD1α-epitope oligosaccharides. From these findings, we propose the oligosaccharide of GD1α is an epitope of an adhesion molecule involved in the interaction of the metastatic tumour cells and the target tissue.

S23

Sialic Acid Requirements for the Interaction of Murine CD22 with CD45

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Low affinity binding kinetics can be studied in real time by biosensor technology. We used this approach to investigate the interaction of purified CD45 with murine CD22, a B cell-restricted molecule, which is a member of the newly defined sialoadhesin family within the immunoglobulin superfamily [1]. CD22 was shown to bind specifically to N-glycans with terminal 2,6-linked sialic acids [2] and has been proposed to bind to CD45 on T cells [3]. In order to investigate the influence of sialic acid linkage and sialic acid modification, CD45 immobilized on the biosensor chip was treated with sialidase, followed by purified sialyltransferases in the presence of either CMP-Neu5Ac or CMP-Neu5Gc as donor substrates. The incorporation of sialic acids was monitored by binding of the linkage specific lectins from *Maakia amurensis* (2,3-specific) and from *Sambucus niger* (2,6-specific). Binding of murine CD22 to CD45 was only observed, if the latter contained Neu5Gc 2,6-linked to N-glycans. No binding could be detected if Neu5Gc was incorporated in the 2,3-linkage or if Neu5Ac was used in either linkage. In conclusion, the interaction of murine CD22 with CD45 is sialic acid dependent and is regulated by the conversion of CMP-Neu5Ac to CMP-Neu5Gc by the corresponding hydroxylase. Furthermore, the modification of glycan structures by successive enzyme treatments on a biosensor chip is a very useful method for the rapid analysis of carbohydrate-binding protein specificities.

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2. Powell LD, Sgroi D, Sjoberg ER, Stamenkovic I, Varki A (1993) *J Biol Chem* **268**: 7019–27.
3. Stamenkovic I, Sgroi D, Aruffo A, Sy MS, Anderson T (1991) *Cell* **66**: 1133–44.

S23

Glycosphingolipids in Lens Tissues

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[3] *Department of Life Science, Tokyo Institute of Technology, Yokohama 227, Japan.*

We have purified neutral and acidic glycosphingolipids (GSLs) from human cataractous lenses and identified Lewis^x (Le^x) and sialyl-Le^x epitopes in neolacto-series GSLs. The age-dependent, cataract-related accumulation of Le^x GSL in lens tissue may enhance cell adhesion between multiple layers of fibre cells by homophilic Le^x-Le^x interaction, leading to the onset and formation of human senile cataract. However, no Le^x GSL was detected in cataracts of experimental animals such as galactosemic rats and hereditary cataractous Emory mice. Among the several mammalian lenses examined, humans and Old World monkeys showed similar GSL compositions, in particular the presence of Le^x and sialyl-Le^x epitopes, in lens tissue. Le^x and sialyl-Le^x epitopes were not observed in lens epithelia, suggesting that the expression of these epitopes in primate lenses was associated with the differentiation of epithelial cells to fibres.

S23

Adhesion of Ovarian Cancer Cells to Hyaluronate: Possible Involvement in Metastasis

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A critical step in the spread of ovarian cancer is the attachment of tumour cells to the mesothelial lining of the peritoneal cavity. It has previously been shown that many ovarian tumour cells express the adhesion molecule CD44 [1], and that mesothelial cells synthesize a pericellular coat that contains hyaluronate (HA) [2]. In this study we measured the adhesion of four ovarian tumour lines (OVMZ6, OVMZ10, OAW59 and O180D) to HA coated on to the surface of a multiwell plate.

This was carried out using a newly developed fluorescent adhesion-assay [3]. Two of the lines (10 and 59), that expressed high amounts of CD44, bound strongly to HA; a third line (6) which was a high CD44 expressor bound moderately well; and the fourth line (180) which did not express CD44 did not bind to the HA. Pretreatment of the HA coat with hyaluronidase, or the presence of soluble HA or an anti-CD44 antibody in the well considerably reduced the adhesion of lines '10 and 59'. These effects were either very small or not seen in the other two lines. These results suggest that the interaction of CD44 with HA may be important in the spread of some ovarian cancers.

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S23

Structures of *N*-linked Oligosaccharides, Sequences of Glycosylation Sites and cDNA Cloning of Vitronectins

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Vitronectin (VN) is a major plasma glycoprotein that has been implicated in a number of physiological and pathophysiological processes. VN may play a role in cell adhesion via integrin receptors and also regulate various biological systems including the blood coagulation, fibrinolysis and complement systems. We previously described the complete oligosaccharide structures of human and porcine VNs (hVN and pVN) [1, 2]; however, the actual glycosylation sites of those VNs were unclear. In this study, we elucidated the glycosylation sites of hVN and pVN together with the cDNA sequence of pVN.

Three *N*-glycosylated sequences were found on hVN by glycopeptide sequence analyses. They coincided with the three potential *N*-glycosylation sites deduced from the cDNA sequence, which are highly conserved among human, mouse and rabbit VN cDNAs. In contrast, only two kinds of *N*-glycosylated sequences were found for pVN. cDNA clones coding for pVN were isolated from a porcine liver cDNA library (lgt 10). The amino acid sequence deduced from the pVN cDNA contained 440 amino acids and 19 amino acids of signal peptide, and showed 69.3% homology with that of hVN. The size of the pVN polypeptide was calculated to be 50 543 Da. Two heparin-binding consensus sequences and two potential *N*-glycosylation sites were found in the sequence, indicating that all the potential sites were actually glycosylated in the mature VNs. The possibility is discussed that several activities of VN can be ascribed to its glycan moieties.

1. Kitagaki-Ogawa H *et al.* (1991) *Glycoconj J* **8**: 224.
2. Yoneda A *et al.* (1993) *Eur J Biochem* **218**: 797–806.

S24. PLANT LECTINS

Chairs: Irwin Goldstein, Avadesha Surolia

S24. 9.50am

An Extensive Sequence Homology Exists in the Primary Structures of Lectins from Different Plant Families, PWM (Phytolaccaceae), WGA (Gramineae) and UDA (Urticaceae)

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One of the most potent plant mitogens was isolated from the root of *Phytolacca americana* (pokeweed or pigeon berry), pokeweed mitogen (PWM). Five mitogens, designated Pa-1 to Pa-5, were separated from pokeweed roots. Pa-1 is mitogenic both to B- and T-cells and Pa-2~5 are mitogenic only to T-cells. Hexa-*N*-acetylchitohexaose was found to interact strongly with these lectins. We demonstrated that Pa-1, Pa-2 and Pa-4 had a strong affinity for highly branched poly-*N*-acetyllactosamine-type carbohydrate chains and also indicated that the specific binding of the isolectins to these carbohydrate chains was due to the lectin's specificity towards the GlcNAcβ1-6Gal sequence.

To elucidate the structural basis for the carbohydrate specificity and mitogenic activity of PWM isolectins, we determined the complete amino acid sequence of Pa-4 using a protein sequencer. The obtained primary sequence of Pa-4 was found to have an extensive sequence homology on a domain-by-domain basis with those of wheat germ agglutinin (WGA), barley lectin (BL), stinging nettle lectin (*Urtica dioica* agglutinin, UDA), and with the amino acid sequences of the N-terminal chitin-binding domains of several chitinases and hevein. It is particularly interesting that plant lectins from different plant families (Phytolaccaceae for Pa-4, Gramineae for WGA, and Urticaceae for UDA) possessed homologous primary sequences.

S24. 10.20am

Exploring The Molecular Features of Lectins and Galectin-Sugar Recognitions

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Despite L-fucose being non-inhibitory, WBA II strongly prefers terminally mono-fucosylated sugars with fucose linked α1-2 to the non-reducing end galactose. 2′-Fucosyllactose is the most complementary ligand. Type I (LNF I) and III H-antigenic structures are poorly recognized. L-Fucose in α1-3 linkage to the penultimate glucose as in 3-fucosyllactose, difucosyllactose and α1-4 and α1-3 linked fucose to *N*-acetylglucosamine as in LNF II and LNF III, respectively, sterically prevent the access of these sugars to the binding site. WBA II exhibits positive entropy change for the binding of 2-fucosyllactose indicating for the first time the predominance of non-polar forces in protein-sugar recognitions. Observation of enthalpy-entropy compensation together with marginal changes in heat capacities for these

interactions suggest that the reorganization of water plays an important role in these recognitions. In other words lectins recognize sugars as amphiphilic molecules. Relative contributions of the polar and non-polar loci of 2-fucosyllactosamine for binding to WBA II will also be discussed. Galectins also seem to recognize their complementary ligands in a similar fashion.

S24. 10.40am

Changes in Con A Structure as a Function of Metal Ion Binding: an X-ray Crystallographic Study

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Concanavalin A (Con A) was the first leguminous lectin pioneered for its three-dimensional structure. The lectin binds a transition metal ion (generally Mn^{2+}) and a calcium ion in neighbouring sites. Sequential binding of the two metal ions is required for the saccharide binding site to be generated.

The 2.5 Å resolution structure of the demetallized lectin has now been unravelled and reveals large conformational differences compared to metal-bound Con A. A non-proline peptide isomerization is presumed responsible for the slow reversible transconformation between the inactive, demetallized lectin and the saccharide binding, active Con A.

To scrutinize further the lectin's activation mechanism and its dependence upon metal ion binding and pH, intermediate steps in the transconformation event are under investigation by means of a series of crystallographic structures. Among those are Con A structures with only one transition metal ion bound (Zn^{2+} or Co^{2+}), obtained by soaking demetallized Con A crystals as well as by cocrystallization, and structures of Con A crystallized at different pHs with both metal ion sites occupied (Zn^{2+} and Ca^{2+}). Also the diversity in metal ion binding specificity is explored: while the substitution of a Mn^{2+} ion by another transition metal ion in mono- and disubstituted Con A is a recurrent theme in nature, the substitution of the metal ion in the more specific Ca^{2+} binding site by Mn^{2+} and Cd^{2+} is a highly unusual result. Therefore the identity of the metal ions in dimanganese Con A will be confirmed in a Multiple Wavelength Anomalous Dispersion experiment.

This work was supported by the V.L.A.B. biotechnology project of the Flemish government. R. Loris is a research assistant of the N.F.W.O.

S24. 11.00am

Sialylated Oligosaccharide-Specific Plant Lectin from Japanese Elderberry (*Sambucus Sieboldiana*) Bark Tissue has a Homologous Structure with Type-II Ribosome Inactivating Proteins, Ricin and Abrin: cDNA Cloning and Molecular Modelling Study

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[3]*National Institute of Forestry and Forest Product Research, Ibaraki, Japan.*

Bark lectins from the elderberry species belonging to the genus *Sambucus* have the binding specificity towards sialylated glycoconjugates containing Neu5Ac(α2-6)Gal/GalNAc sequence [1] and have been a useful tool for the detection and isolation of sialylated glycoconjugates. The elderberry bark lectin is a tetrameric glycoprotein consisting of two different subunits, hydrophobic and hydrophilic subunits, and has two carbohydrate binding sites in the molecule [1, 3]. To elucidate the structure of Japanese elderberry (*S. sieboldiana*) bark lectin (SSA, [2, 3]), a cDNA library was constructed from the mRNA isolated from the bark tissue using λgt 11 phage and screened with anti SSA antibody. The nucleotide sequence of a cDNA clone encoding full length SSA showed the presence of an open reading frame with 1904 bp which corresponded to 564 amino acid residues. This open reading frame encoded both of two subunits of SSA, 'linker' region (19 amino acid residues) between two subunits, and a signal peptide (28 amino acid residues). This indicates that SSA is synthesized as a preproprotein and post-translationally cleaved into two mature subunits. Homology search as well as molecular modelling studies unexpectedly revealed that each subunit of SSA has a highly homologous structure with the galactose-specific lectin subunit and ribosome inactivating subunit of plant toxic proteins such as ricin and abrin, indicating a close evolutional relationship between these carbohydrate binding proteins.

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S24. 11.30am

Site-Directed Mutagenesis of the Lima Bean Lectin Results in Altered Carbohydrate Recognition

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The specificity of legume lectins for known oligosaccharide structures has made them useful tools in the study of cell surface glycoconjugates. The wild type seed lima bean lectin (LBL) and recombinant LBL expressed in *E. coli* show specificity for the human blood group A immunodominant trisaccharide GalNAcα1-3[Fucα1-2]Galβ1-R. We have generated four site-specific mutants of the lima bean lectin, two of which show altered specificity for extended carbohydrate structures. The mutants, [C127Y]LBL, [H128P]LBL, [H128R]LBL and [W132F]LBL were expressed in *E. coli*. Two mutants show altered specificity for the substituent at the C2 hydroxyl group of the penultimate Gal in the wild type ligand which is α-L-fucose in the A trisaccharide. Mutant [C127Y]LBL showed specificity for the A disaccharide (GalNAcα1-3Gal) and GalNAcα1-4Gal, with free hydroxyl groups at the C2 position of Gal. Mutant [H128P]LBL bound the Forssman disaccharide structure (GalNAcα1-3GalNAc) in which the C2 hydroxyl group is substituted with an acetamido group. Mutants [H128R]LBL and [W132F]LBL exhibited wild type specificity, both recognizing the A trisaccharide. All of these mutant lectins bound the terminal GalNAc residues exposed on asialoovine submaxillary mucin, indicating that the monosaccharide binding site had not been altered. Thus, by targeting two residues in the primary structure of LBL, we have identified a region of the protein that is part of the extended carbohydrate binding site, and specifically involved in the binding/recognition of substituents at the C2 position of the penultimate Gal of the A

disaccharide. We have determined by site-directed mutagenesis that an essential cysteine is involved in the specificity of the lima bean lectin for the A trisaccharide.

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S24. 11.50am

Comparison of Con A and Anti-α-D-Glucose Antibodies

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Concanavalin A (Con A), a plant lectin, is a glycoprotein with remarkable ability to bind to carbohydrate units of macromolecules. The lectin has been used in a variety of biological tests such as a mitogen for lymphocytes, an anti-cancer drug for tumours, an agent for differentiating normal and malignant cells and a model system for antibody-antigen reactions. The structural units of macromolecules which react with Con A are terminal α-D-glucose (α-Glc) or α-D-mannose units. Reactivity of Con A with α-D-Glc units has now been found to occur with a glycoconjugate, α-Glc-bovine serum albumin. Anti α-Glc antibodies have been isolated from serum of rabbits immunized with the glycoconjugate. A comparison of properties and reactivity of Con A and the anti-α-Glc antibodies has been made. The molecular weight, the protein type, gel electrophoretic mobility, isoelectric point and the inhibition of Con A and α-Glc antibodies by glucose derivatives have been determined. The antibodies only react with anti-IgG serum but Con A reacted with anti-IgA, anti-IgG and anti-IgM serum. A variety of glucose derivatives were tested as inhibitors for the precipitin reactions. The inhibition results are interpreted to show the points of binding of glucose of macromolecules to Con A and α-Glc antibodies. For binding of glucose units to occur to Con A, the glucosidic oxygen in the α form, hydroxyl groups at C_3 and C_4 in the equatorial orientation, the pyranose ring form and a hydroxyl group at C_6 are necessary. The above structural features and the hydroxyl group at C_2 are involved in the binding of the glucose units to the anti-α-Glc antibodies.

S24 POSTERS

S24

Use of Lectins to Reconstitute Membrane Glycoproteins into Lipid Vesicles. Application to VIP Receptor

Assou El Battari, Jan Chochola, José Luis, Catherine Bellan, Monique Roccabianca and Jacques Marvaldi
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We describe here a rapid method for reconstituting hydrophobic glycoproteins into lipid vesicles. This technique involves aggregation of detergent solubilized glycoproteins with wheat germ agglutinin (WGA) or *Datura Stramonium* agglutinin (DSA) and displacement of the lectin with the competing trisaccharide *N*,*N*′,*N*″-triacetylchitotriose (TAC). When the pellet is resuspended in the absence of any detergent but in the presence of TAC, soluble glycoproteins are released in the medium whereas the insoluble material could be pelleted by ultracentrifugation. Routinely, the final pellet contained 3–5% of total protein. Examination of this pellet by electron microscopy showed the presence of multilamellar lipid vesicles (MLV). We applied this method to the VIP receptor, a membrane glycoprotein with seven putative hydrophobic transmembrane domains, and found that all ^{125}I-VIP-receptor complexes could be pelleted from n-octyl-β-D-glucoside (OG) extract. The reconstitution of functional VIP receptors was also successfully achieved. Use of the lectin aggregation technique to reconstitute glycoprotein rich membranes is an improvement over the use of other techniques because it is rapid, specific, reversible and non denaturant. The presently described preparation should be useful for reconstituting and studying solubilized hydrophobic glycoproteins such as receptors and ion channels.

S24

Tricophyton rubrum Dermatophyte Agglutinin is Apparently an 'Endolectin' In Nature

Aryya Mitra, Bishnu P. Chatterjee and *Arun K. Guha*
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Tricophyton rubrum agglutinin (TRA) from 3-week-old culture filtrate has been purified to homogeneity by affinity chromatography on a fetuin Sepharose column. The homogeneity of the agglutinin has been tested by several biochemical as well as immunochemical techniques. TRA has a molecular weight of 6.5 kDa and is a human blood group non-specific agglutinin and besides human, it agglutinates several animal erythrocytes. The erythroagglutinating activity has not been inhibited by mono- and oligosaccharides including free sialic acid, human HOG, several galactomannans, colominic acid and GT_{1b} ganglioside. Whereas several sialic acid containing glycoproteins *viz*, fetuin, ceruloplasmin, bird's nest glycoprotein, porcine thyroglobulin, human glycophorin, porcine stomach mucin, γ-globulin, α_2-macroglobulin, α_1-acid glycoproteins, human transferrin and 9.5 s glycoprotein are inhibitors of TRA. Among the glycoproteins tested, fetuin has been found to be the most potent inhibitor whereas asialo derivatives of some of the glycoproteins except fetuin are non-inhibitory. From the above study, the endolectin nature of TRA is discussed in the light of inhibitory potency of sialic acid containing oligosaccharides having different linkages and sugar sequences.

S24

Sialic Acid-Binding Motifs of *Maackia amurensis* Lectins

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Maackia amurensis hemagglutinin (MAH) and leukoagglutinin (MAL) are leguminous lectins which recognize carbohydrate chains containing sialic acid residues. In the present investigation cDNA clones encoding for MAH and MAL were isolated from a cDNA library constructed from germinated *Maackia*

amurensis seeds, and sequenced. From the reading frame of the cloned cDNAs, MAH and MAL are predicted to be composed of 285 and 287 amino acid residues, respectively. In leguminous lectins, most of amino acid residues involved in sugar-binding are known to be conserved. However, in MAH and MAL, amino acid residues corresponding to the conserved Gly and Asn were substituted by Lys-105 and Asp-135, respectively. To assess the importance of these amino acids in carbohydrate recognition, they were substituted by recombinant technology. Mutant lectins with Lys-105 to Gly and/or Asp-135 to Asn did not bind to sialic acid-containing glycopeptide CB-II from human glycophorin A. These results indicated that these residues played an important role in sialic acid-binding of MAH.

THURSDAY 24 AUGUST, AFTERNOON

PLENARY LECTURES

S25. 2.10pm

Recognition of Target Cell Glycoconjugates by Protozoan Parasites

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Intracellular protozoan parasites may have restricted or promiscuous target cell specificity. Malaria sporozoites injected into the blood develop exclusively inside hepatocytes, while *Trypanosoma cruzi*, the causative agent of Chagas disease, develop in most cell types. In both instances, recognition of glycoconjugates from the plasma membrane of the host's cells plays a central role in the attachment phase of invasion. *T. cruzi* infective stages express on their surface a singular enzyme, a trans-sialidase. During *T. cruzi* invasion, this enzyme recognizes sialylated molecules on the plasma membrane of target cells, and transfers this sialic acid onto mucin-like molecules of the parasite surface. The plasma membrane of malaria sporozoites is covered by the circumsporozoite protein (CSP). When injected into mice, CSP attaches only to the glycosaminoglycan (GAG) chains of heparin sulfate proteoglycans (syndecans?) from the basolateral membrane of hepatocytes. This is the probable explanation of the strict specificity of sporozoites for liver cells. Recent evidence suggests that the physiological role of the GAG receptors for the malaria CSP is to sequester very low density lipoprotein remnants and chylomicron remnants from the blood circulation.

S26. 2.40pm

Selectin Oligosaccharides and Sphingosine Derivatives in Reperfusion Injury

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Selectins have been found to mediate early leukocyte-endothelial interaction in reperfusion injury. P-selectin is maximally up-regulated on the surface of the coronary vascular endothelium 10–20 min post-reperfusion. In this context, a variety of anti-selectin agents have been tested in myocardial ischemia-reperfusion in cats. The following table lists the major substances tested and the basic results obtained:

Agent tested	*Inhibition of PMN adherence to EC*	*Inhibition of infiltration*	*Attenuation of myocardial necrosis*
Anti-P selectin MAb	+++	+++	+++
Anti-L selection MAb	++	++	++
Anti-E selectin MAb	0	0	0
Sialyl Lewis^x OS	++++	++++	++++
Non-sialylated control OS	0	0	0
Liposome conjugated (SLe^x-OS)	++++	++++	++++
Tri methyl-sphingosine (TMS)	+++	+++	+++

These data indicate that L- and P-selectin, but not E-selectin are critically involved in PMN interaction with the endothelium (EC) which is a prerequisite for reperfusion injury. SLe^x-oligosaccharides (OS) which may block more than one selectin are even more effective. Sphingosine analogues (eg, TMS) which inhibit cell signalling pathways essential for selectin activity are also effective agents in treating reperfusion injury.

S27. 3.10pm

Metabolic Fate and Functional Implications of Exogenous Glycosphingolipids in Neural Cells

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The addition of glycosphingolipids, particularly gangliosides, to cells in culture is capable of modifying the functional behaviour of the same cells. Cells of neural origin, like neuroblastoma, pheochromocytoma, and sensory ganglion cells, respond to exogenously added gangliosides by undergoing differentiation with formation of neurite- or dendrite-like processes. Under

these conditions, exogenous gangliosides interact with various components of the cell plasma membrane and insert into the external membrane layer, thus behaving as their endogenous counterparts. Therefore, some of the induced effects, mimicking a physiological situation, derive from a ganglioside influence on functional membrane proteins (ion channels and pumps, enzymes, receptors, etc.) involved in cell-cell interactions and transmembrane signalling events. Moreover, taken-up gangliosides are promptly endocytosed, introduced into the intracellular membrane trafficking and degraded in the lysosomal apparatus, with formation of metabolites, including ceramide and sphingosine. These compounds belong to a family of bioactive molecules playing a role in cell growth, differentiation and apoptosis. Thus the additional possibility exists that glycosphingolipids exert functional roles, by producing bioregulators of a sphingoid nature.

S25. GLYCOPATHOLOGY OF PARASITES

Chairs: Roger Laine, Salvatore Turco

S25. 4.00pm

Developmentally Modified LPG on Infective Stages of *Leishmania* Promastigotes

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The life-cycle of *Leishmania* parasites within the sandfly vector includes the development of extracellular promastigotes from a non-infective, procyclic stage into an infective, metacyclic stage which is uniquely adapted for transmission by the fly and survival in the vertebrate host. These adaptations were explored in the context of the structure and function of the abundant surface LPG of *Leishmania* promastigotes. During metacyclogenesis, the salient structural feature of the *L. donovani* LPG is conserved, involving expression of a phosphoglycan chain made up of unsubstituted disaccharide-phosphate repeats. Two important developmental modifications were also observed. First, the size of the molecule is substantially increased due to an approximate three-fold increase in the number of phosphorylated disaccharide repeat units expressed. Second, there is a down regulation of terminally exposed capping sugars on metacyclic LPG, resulting in the loss of lectin binding sites for both peanut agglutinin and concanavalin A, as well as sugar substrate for galactose oxidase. Refined structure analysis revealed that despite the loss of terminal sugars available for binding, metacyclic LPG continues to express several neutral capping oligosaccharides which terminate in either β-linked galactose or α-linked mannose. The masking of these terminal sugars is attributed to a change in conformationl structure associated with folding and clustering of the extended phosphoglycan chains, which form densely distributed particulate structures visible on fracture-flip preparations of the metacyclic surface. The exposure and subsequent masking of the terminal capping sugars were shown to control the stage-specificity of promastigote attachment and release from the sandfly midgut, which are key events in the development of transmissible infections.

S25. 4.10pm

Interaction Between the Malarial Parasite and the Erythrocyte

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The erythrocytic stage of a malarial infection is characterized by a specific and rapid invasion of the erythrocyte by the malarial merozoite. This process may involve initial recognition/binding followed by a cascade of events culminating in invasion. Prior reports have implicated glycophorin as one participant in the recognition process involving *P. falciparum* merozoites and the human erythrocyte. Using a combination of monoclonal antibodies and peptide fragments we have mapped the locus on glycophorin primarily responsible for parasite recognition. This sub-domain, residues 10–15, is characterized by dense glycosylation, high sialic acid content and correspondingly high net negative charge. These data are consistent with the reported resistance of sialic acid deficient erythrocytes to invasion by the *P. falciparum* merozoite and inhibition of invasion by oligodeoxynucleotides, heparin and related highly anionic carbohydrates such as pentosan polysulfate and fucoidan. An invasion-inhibitory monoclonal antibody whose recognition is sialic acid dependent and which binds to the identified sub-domain has been sequenced and found to have significant homology to two regions of the major surface protein of the merozoite. In addition, an anti-idiotype antibody developed against this monoclonal specifically recognizes a carboxyl terminal fragment of the parasite protein which contains these same homology domains; the anti-id is also capable of inhibiting invasion.

Supported by Grant N-00014-90-J2032 from ARPA.

S25. 4.20pm

Polylactosamines are not Obligate Receptors for Invasion of *Plasmodium falciparum* Malaria as Shown in HEMPAS Variant II-(gal-)erythrocytes

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An example of HEMPAS Variant II(gal-) erythrocytes was characterized by analysis of the total RBC carbohydrates. Polylactosamines were completely lacking from both erythrocyte glycoprotein and glycolipids as assessed by gel permeation chromatography of released carbohydrates. Malarial invasion of these erythrocytes was lower, but not precluded by the complete lack of cell surface polylactosamines. The life cycle of merozoites was not affected by microscopic analysis of interme-

diate forms. Since the lack of glycophorins on the erythrocyte has been shown not completely to block invasion by falciparum malaria by a number of groups, we must conclude that neither glycophorins, their carbohydrates, nor polylactosamines are obligate receptors for parasitic invasion.

S25. 4.30pm

Glycopathology of *Trypanosoma Cruzi*

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The parasitic protozoan, *Trypansoma cruzi*, is the causative agent of Chagas' disease, which results in cardiomyopathy and neuropathy. Chagas' disease is endemic in Latin American countries and is becoming epidemiologically important in the United States. This obligate intracellular parasite has a complex life cycle, with multiple development stages in the insect vector and mammalian host. Glycoconjugates on the surface of *T. cruzi* most likely play a key role in host-parasite interactions. Our research is currently focused on *T. cruzi* cell surface glycoconjugates and parasite's binding (adhesion) to glycolipid receptors of host target tissues. Structural and receptor binding studies should provide knowledge crucial to understanding the biochemical/molecular mechanisms of glycopathology of *T. cruzi* parasite. The novel sialylated, 38/45 kDa glycosylated phosphatidyl-inositol (GPI) anchor molecule from *T. cruzi* epimastigotes has been isolated, purified and partially characterized. This molecule is anchored on the cell surface via an alkylacylphosphatidyl-inositol. Results also suggest the presence of a peptide containing large numbers of threonine residues covalently linked to the 38/45 kDa glycoconjugate. β-Elimination of the molecule showed the presence of GalNAc and GlcNAc, suggesting at least some mucin-like O-linked oligosaccharide(s). Matrix-assisted laser desorption/ionization time-of-flight (MALDI/TOF) mass spectral analysis of the native molecule indicated the presence of two major components whose molecular masses were about 18.4 and 22.5 kDa. Biochemical and immunological studies of the 38/45 kDa glycoconjugate of epimastigotes showed strong similarities to Ssp-3 antigens (GP60-200 kDa) from trypomastigotes. The detailed structure of the 38/45 kDa molecules is under investigation. *T. cruzi* epi-, trypo- and amastigotes each bind to specific classes of glycolipids in a TLC overlay assay. This difference in binding indicates a regulated expression, suggesting the likelihood of receptor-ligand interactions.

S25. 4.40pm

On the Structure and Biosynthesis of the Glycosylphosphatidylinositol Membrane Anchor of the Merozoite Surface Proteins-1 and -2 of *Plasmodium falciparum*

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Plasmodium falciparum accumulates the two merozoite surface proteins (MSP)-1 and -2 during schizogony. Both proteins are proposed to be anchored in membranes by glycosylphosphatidylinositol (GPI) membrane anchors. The identity of these GPI-anchors is confirmed by labelling with tritiated precursors and additionally by specific enzymatic and chemical treatments. Detailed structural analysis of the core-glycans showed that the GPI-anchors of both proteins possess an extra α,1-2 linked mannose at the conserved trimannosyl-core-glycan.

Thus, of the two candidates for putative GPI-anchor precursors to malarial membrane proteins with the structures ethanolamine-phosphate-6(Manα1-2)Manα1-2Manα1-6-Manα-1-4GlcN-PI ($Pf_{gl}\alpha$) and ethanolamine-phosphate-6Man α1-2Manα1-6Manα1-4GlcN-PI ($Pf_{gl}\beta$) only $Pf_{gl}\alpha$ seems to be involved in the post-translational modification of MSP-1 and MSP-2. The inositol ring in both GPI-anchors is modified primarily by myristic acid, until now a unique feature for all described GPIs. Additionally the hydrophobic fragments were identified as diacyl-glycerols, carrying preferentially palmitic acid in an ester-linkage.

Furthermore, we investigated the proportions of $Pf_{gl}\alpha$ and $Pf_{gl}\beta$ formed during the asexual intraerythrocytic development of the parasite. Significant differences between early and late stages were found.

S25. 4.50pm

Structure and Biosynthesis of the Secreted Proteophosphoglycans of *Leishmania* Parasites

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Protozoan parasites of the genus *Leishmania* secrete a heavily glycosylated acid-phosphatase and a number of related proteoglycans. In *L. mexicana*, these molecules form long filamentous heteropolymers or fibrous networks that bind the parasites together in clusters. They are secreted by all developmental stages of the parasites and may play a role in parasite survival in the digestive tract of the insect vector and in the phagolysosome of mammalian macrophages. We have recently shown that the protein backbone of these molecules contain serine-rich domains that are heavily substituted with short chains of phosphorylated di- or tri-saccharide repeat units and/or mannose oligosaccharides [1]. All of these structures are linked to the polypeptide backbone via a Manα1-PO_4-Ser linkage. We have synthesized a range of serine-rich peptides which are similarly glycosylated in a cell free system. The glycosylated peptides have been extensively characterized by electrospray mass spectrometry, Edman sequencing and analysis of the acid-released glycans. These peptides are readily substituted with Man-1-PO_4 but not with the more elaborate glycan structures found on the native glycoproteins/proteoglycans. This substitution appears to be initiated by an enzyme that utilizes GDP-Man as donor and transfers Man-1-PO_4 directly to the Ser residues. A convenient assay for this enzyme has been developed and its properties investigated. These data suggest that the Man-1-PO_4 transferase involved in proteoglycan biosynthesis may be closely related to a similar enzyme involved in synthesis of the abundant surface lipophosphoglycan of these parasites.

1. Ilg T, Overath P, Ferguson MAJ, Rutherford T, Campbell DG, McConville MJ (1994) *J Biol Chem* **269**: 24073–81.

S25. 5.00pm

The α-Mannosidase of *T. cruzi*

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Chagas' disease (American trypanosomiasis) is caused by the protozoan *Trypanosoma cruzi.* Although carbohydrate structures have been implicated in many host-parasite interactions, the glycobiology of most parasites, including *T. cruzi,* has not been described in detail. We are currently investigating the biochemistry, biosynthesis, glycosylation, and intracellular sorting of a prototypic lysosomal acid hydrolase of *T. cruzi,* α-mannosidase. We have previously reported the purification and characterizaton of the acid α-mannosidase [1]. The α-mannosidase has a native molecular weight of 240 000 Da and is composed of 4 identical subunits (58 000 Da) each of which contains one N-linked high mannose type oligosaccharide. The enzyme has been localized by immunogold to the reservosome of the epimastigote. The preferred substrate(s) is (are) linear (α 1-2) ≫ (α 1-4) ≫≫ (α 1-6) = (α 1-3) oligosaccharides and branched oligosaccharides such as $GlcNAc_2$ $Man_{7\text{-}9}$ are poor substrates. Currently, the α-mannosidase is being cloned in order to study structure/function relationships at the gene level. The α-mannosidase belongs to the family of Class 2 α-mannosidases and bears some sequence homology to mammalian lysosomal α-mannosidases. These studies will enable us to elucidate the general pathway of biosynthesis and sorting of lysosomal enzymes in *T. cruzi* and to establish this aspect of the glycobiology of the parasite.

1. Swanson *et al* (1992) *Glycobiology* **2**: 563–69.

S25. 5.10pm

Substrate Specificity of Trans-sialidases from African Trypanosomes: Transfer of Hydroxylated and 9-*O*-Acetylated Sialic Acids onto Trypanosomal and Synthetic Acceptor Compounds

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In African trypanosomes the expression of trans-sialidases coincides with the sialylation of major parasitic surface glycoproteins [1, 2]. Although the enzymes from *Trypanosoma brucei* and *T. congolense* differ in some properties, e.g. surface localization, both trans-sialidases are enzymatically similar and their biological function seems to be analogous [2, 3]. A comparative analysis of the substrate specificity of the two known African trypanosomal trans-sialidases led to the following conclusions: (i) The substrate specificity of *T. brucei* and *T. congolense* trans-sialidases is essentially identical. (ii) Both enzymes show a strong preference for α(2-3)-linked sialic acids. (iii) Hydroxylation of sialic acid as in Neu5Gc does not affect the transfer rate. (iv) Neu5,9Ac_2-lactose is a donor substrate for both trans-sialidases, however, *O*-acetylated sialic acids are transferred at an 85% slower rate when compared to Neu5Ac or Neu5Gc, respectively. (v) The trypanosomal sialic acid acceptors PARP (*T. brucei*) and GARP (*T. congolense*) are readily trans-sialylated by both enzyme activites. Also hydroxylated and *O*-acetylated sialic acids can be transferred onto the GPI-anchor glycans of PARP and GARP, respectively. (vi) A variety of synthetic compounds, including 4-methylumbelliferyl-β-D-galactoside, 4-nitrophenyl-β-D-galactoside and isopropyl-thio-β-D-galactoside, are potent sialic acid acceptors.

1. Engstler M *et al*. (1993) *Mol Biochem Parasitol* **61**: 1–14.
2. Engstler M *et al*. (1995) *Acta Tropica*, in press.
3. Engstler M, Schauer R (1993) *Parasitol Today* **9**: 222–25.

S25. 5.20pm

Structural Characterization of Novel Glycoinositolphospholipids (GIPLs) From Two Opossum-Derived Strains of *Trypanosoma cruzi*

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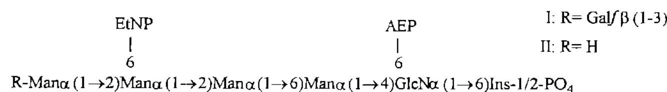
The structures of the cell-surface GIPLs of *T. cruzi* G and G-645 strains were investigated. The major PI-oligosaccharides liberated by treatment with alkali were characterized by classical methods, high field NMR spectroscopy and FAB mass spectrometry and shown to have the structures I and II.



The GIPL from *T. cruzi* G-strain is a mixture of structures I and II in molar ratio of 7:3 whilst the GIPL from G-645 seems to be almost completely structure I. These glycolipids differ from previously characterized LPPG from *T. cruzi* Y-strain [1] in that the third mannose distal to GlcN is substituted with ethanolamine phosphate (EtNP) on O-6 rather than Gal*f*β (1 → 3). These molecules seem to have biological importance since they stimulate immunoglobulin secretions when added to murine B cells in the presence of cytokines.

1. Previato *et al*. (1990) *J Biol Chem* **265**: 2518.

S25. 5.30pm

Heart Cells Share Common Neutral Glycosphingolipids with *Trypanosoma cruzi*

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[3]*Physiologische-Chemisches Institut, Universität Bonn, Germany.*

Infections with *T. cruzi* result in the formation of chronic lesions in many tissues, including muscle and nervous systems. The Chagas disease chronic phase pathology is though to be of autoimmune origin, due to the presence of cross-reactive antigens between the parasite and the mammalian host or due to failure of self/non-self discrimination in the infected host. It has been shown that some glycoconjugates such as glycolipids and glycoproteins are cross reactive antigens between the

parasite and heart muscle cells. Neutral glycosphingolipids were isolated from mouse heart muscle cells and their structures were elucidated using a combination of techniques such as column chromatography, HPTLC, GC and fast atom bombardment mass spectrometry (FAB-MS). Neutral glycosphingolipids from heart muscle cells comprising mainly monohexosyl (CMH) and dihexosylceramides (CDH) were purified by silica and Iatrobeads column chromatography. The CMH fraction contains either glucose and galactose in a 1:1 ratio, sphingosine and as fatty acyl groups mainly C-18 saturated or 2-hydroxy fatty acids. The CDH was identified as a lactosylceramide. Our results clearly demonstrated that *T. cruzi* and heart cells from mouse share very similar lipidic antigens. Both present the same carbohydrate residues differing in the proportion of fatty acids of the ceramide portion. C18:OH was the predominant fatty acid found in heart and C24:OH in *T. cruzi* CMH. These findings suggest that these glycosphingolipids could be a class of cross-reactive antigens. These molecules acting as cross-reactive antigens could participate singly or together with other molecules in the autoimmunity process described in the pathogenesis of Chagas disease.

Supported by Grants from CNPq/PADCT/FNDCT, FINEP and UFRJ.

S25. 5.40pm

Structural Characterization of a New Galactofuranose-Containing Glycolipid Antigen of *Paracoccidioides brasiliensis*

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An acidic glycolipid termed band 1 was isolated from *P. brasiliensis* by a combination of ion exchange chromatography and HPLC. Band 1 was found to be reactive with sera of all patients with paracoccidioidomycosis (PCM). Monosaccharide analysis indicated that the glycan of band 1 contains mannose and galactose. Preliminary analysis of ^1H NMR and mass spectrometry data suggest that the structure of the non-reducing end is Manp$\alpha 1 \rightarrow 6$(Gal$f\beta 1 \rightarrow 3$)Manp$\beta 1 \rightarrow$ R. Presence of galactofuranose in band 1 was determined by mild acid hydrolysis and by mild periodate oxidation and reduction with NaB^3H_4. Removal of galactofuranose residues decreased by 60–80% the reactivity of sera from PCM patients with band 1, suggesting that these residues are immunodominant in band 1 antigen from *P. brasiliensis*.

Supported by PADCT/CNPq, FAPESP, FINEP and CNPq.

S25. 5.50pm

Substrate Specificity of the Dol-P-Man:GlcN-PI a1-4 Mannosyltransferase of the Glycosylphosphatidylinositol (GPI) Pathway of African Typanosomes

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Mannosylation of D-GlcNα1-6D-*myo*-inositol-1-PO_4-*sn*-1,2-dimyristoylglycerol (GlcN-PI) was studied using membrane preparation from the bloodstream forms of African trypanosomes. The mannosyltransferase activity was assayed using GDP-[^3H]Man together with chemically synthesised GlcN-PI to form a variety of radiolabelled GPI intermediates.

Several substrate analogues of GlcN-PI were synthesized and screened to determine the minimum structure required for recognition by the Dol-P-Man:GlcN-PIα1-4 mannosyltransferase. D-GlcNa1-6D-*myo*-inositol was found to be the smallest structure with acceptor activity. The stereochemistry of the inositol ring was deemed to be vital for substrate recognition, since neither D-GlcNα1-6L-*myo*-inositol-1-PO_4-*sn*-1,2-dimyristoylglycerol nor D-GlcNα1-6L-*myo*-inositol showed acceptor activity.

The amino group of GlcN-PI also appeared to be important for acceptor substrate recognition, since Glc-PI and 2-deoxy-Glc-PI were neither acceptors nor inhibitors of the Dol-P-Man: GlcN-PIα1-4 mannosyltransferase.

The donor substrate specificity of the Dol-P-Man:GlcN-PIα1-4 mannosyltransferase was also examined. The dolichol-phosphate analogues isoamyl-phosphate and didehydrofarnesol-phosphate were synthesised and found to be substrates for the trypanosome Dol-P-Man synthetase. However, the Dol-P-Man:GlcN-PIα1-4 mannosyltransferase was unable to use isoamyl-phosphate-mannose or didehydrofarnesol-phosphate-mannose as donor substrates.

S25. 6.00pm

Infection of Monkeys and Humans with the Helminthic Parasite *Schistosoma mansoni* Induces Autoimmunity to the Lewis x Antigen

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We reported previously that adult *S. mansoni* synthesize a group of high molecular weight glycoproteins with complex-type N-linked oligosaccharides bearing the fucose-containing Le^x antigen [1]. Oligosaccharides containing the Le^x structure bound specifically to immobilized sera from *S. mansoni* infected hamsters. We now report our discovery that antibodies to Le^x antigens are present in sera from *S. mansoni* infected mice, hamsters, rhesus monkeys and humans. The presence of anti-Le^x antibodies was determined by an ELISA using the Le^x containing neoglycoprotein, lacto-*N*-fucopentaose-III-BSA (LNFP-III-BSA) as target. We observed that infected animals had high titres of both IgM and IgG antibodies reactive to LNFP-III-BSA. Sera from *S. mansoni* infected humans contained only IgM antibodies reactive to LNFP III-BSA and the titre was lower than observed for infected animals. These antibodies to Le^x were lytic and mediated specific complement lysis of the human promyelocytic leukaemia cell line HL-60, which bear surface Le^x determinants. These results demonstrate that an autoimmune disorder based on reactivity to Le^x antigens accompanies infection with *S. mansoni*.

Supported by NIH Grant A126725 to R.D.C.

1. Srivatsan *et al.* (1992) *J Biol Chem* **267**: 20196–203.

S25. 6.10pm
Discussion

S25 POSTERS

S25
Developmental Lipid Modification of Sialic Acid Acceptors of *Trypanosoma cruzi* During Differentiation of Epimastigotes into Infectives Metacyclic Trypomastigotes

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Trypanosoma cruzi is unable to synthesize sialic acid (SA) but expresses an unique *trans*-sialidase on its cell surface which transfers SA residues from host glycoconjugates to terminal β-galactosyl residues-containing acceptors present on the parasite surface. The natural acceptors of sialic acid in both non-infective (epimastigotes) and infective (metacyclic trypomastigotes) forms are mucin-like glycoproteins linked to the parasite surface via glycosylphosphatidylinositol (GPI) anchors. To better understand the function of SA acceptors in these two parasite stages, we deduced the structure of GPI anchor and O-linked oligosaccharides of these molecules. The only difference found, between the infective and non-infective stages, was at the lipid portion. While the phosphatidylinositol moiety of the epimastigote mucin is formed by 1-*O*-hexadecyl-2-*O*-hexadecanoyl-phosphatidylinositol, the metacyclic mucin is mostly composed of inositol-phosphoceramide, consisting of $C_{18:0}$ sphinganine and $C_{24:0}$ and $C_{16:0}$ as major fatty acid species. The GPI glycan cores were identical in both mucins and it was formed by Manα1-2Manα1-2Manα1-6Manα1-4GlcNH. The O-linked oligosaccharides of metacyclic mucins were also analysed and the deduced structures were identical to those reported in the mucin of epimastigotes [1]. We concluded that, during metacyclogenesis, the phosphatidylinositol moiety of the major acceptor of sialic acid is modified, while the sugar chains are highly conserved. These lipid changes probably reflect differences in the GPI biosynthetic pathway between these parasite stages, and might be related to parasite infectivity.

1. Previato *et al.* (1994) *Biochem J* **301**: 151–59.

S25
Carbohydrate Derivatives as Substrates for Glycosyltransferases in *Trypanosoma brucei*

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Studies in these laboratories are aimed at purifying and characterizing the enzymes involved in glycosylphosphatidylinositol (GPI) biosynthesis in African trypanosomes. The GPI biosynthetic pathway in bloodstream form African trypanosomes is thought to be a potential target for chemotherapeutic treatment of trypanosomiasis. The natural substrates for the GPI biosynthetic enzymes are highly complex structures that require lengthy chemical syntheses. Therefore, as part of our work, simple substrate analogues are being investigated. Simple thio-octyl glycosides have been synthesized and tested for their ability to act as acceptor substrates for trypanosome glycosyltransferases of the GPI biosynthetic pathway and of the dolichol-cycle.

S25
Carbohydrate Moiety of *Plasmodium falciparum* 195 kDa Glycoprotein

Nasir-ud-Din, D. C. Gowda, A. P. Torres-Duarte and E. A. Davidson
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Metabolic labelling of the FCR-3 strain of *Plasmodium falciparum* parasites with tritiated glucosamine and subsequent purification by SDS-PAGE of the parasitic material provided effective labelling of the 195 kDa, MSP-1, glycoprotein. The parasitic cells were labelled in synchronous and asynchronous modes. There was diversity of sugar incorporation in different glycoproteins; the 195 kDa glycoprotein incorporated significantly more glucosamine than mannose, whereas galactose incorporation was weak and fucose incorporation was not observed. In control experiments containing erythrocytes, no detectable labelling of erythrocytic proteins was observed. The results of monosaccharide analysis, reductive β-elimination with sodium hydroxide-sodium borohydride, glycosidase treatment, lectin binding, and exogalactosylation of the metabolically labelled 195 kDa protein suggest that in this glycoprotein: (i) the carbohydrate chains are attached to the protein backbone primarily if not exclusively via *O*-glycosyl linkages; (ii) the carbohydrate moiety mainly consists of short chains linked to the protein core; and (iii) a small amount of label appears in the GPI anchor.

Malaria is the most prevalent parasitic disease; the native 195 kDa protein confers significantly more immune protection than recombinant chimeric proteins.

Supported by Grant N-00014-90-J2032 from ARPA and funds from WHO.

S25
Structural Analysis of Glycolipids of the Parasitic Nematode *Ascaris suum*

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In order to assess the potential involvement of parasitic nematode glycolipids in mechanisms promoting an evasion of the host defence system, we are studying the chemical, immunological and biological properties of parasitic nematode-derived glycolipids using *A. suum* as a model.

Glycolipids were extracted from homogenized worms and fractionated according to charge by anion-exchange chromatography. Zwitterionic and neutral glycolipid species present were separated by chromatography on silica gel and by preparative HPLC yielding four neutral and four zwitterionic fractions. Individual glycolipid compounds were analysed by LSIMS,

MALDI-MS, methylation analysis and exoglycosidase digestion. For immunological characterization, polyclonal antibodies directed against zwitterionic species were used for localization as well as characterization of the glycolipid-bound antigenic epitope(s).

The results revealed that the carbohydrates of *A. suum* neutral glycolipids reflect the arthro-carbohydrate series which had been, so far, detected solely in insects (phylum Arthropoda) [1]. Zwitterionic glycolipids were shown to contain phosphocholine as the major antigenic epitope. Detailed structural analyses of their carbohydrate and lipid moieties as well as the precise assignment of the phosphocholine residue are in progress.

1. Dennis RD, Wiegandt H (1993) *Adv Lipid Res* **26**: 321–51.

S25

Monoclonal Antibody (MEST-1) Directed to Galactofuranose Residue of a Glycolipid Antigen of *Paracoccidioides brasiliensis*

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A mouse monoclonal antibody MEST-1 (IgM) was raised against band 1 glycolipid antigen of yeast forms of *Paracoccidioides brasiliensis*. By solid-phase radioimmunoassay (RIA) it was determined that MoAb MEST-1 is able to detect as low as 5 ng of purified band 1. By solid-phase RIA and HPTLC immunostaining, it was shown that reactivity of MEST-1 with band 1 is abolished after mild treatment with sodium m-periodate (conditions to oxidize only furanose forms) followed by reduction with $NaBH_4$. These data indicate that the galactofuranose residue is recognized by the MEST-1 antibody. Confirming this result, antibody binding to band 1 was effectively inhibited in solid-phase RIA only by β-D-methyl-galactofuranose (25 mM inhibit 100% of binding). No inhibition was observed with other methylglycosides. MEST-1 is the first mouse MoAb described to react specifically with β-D-galactofuranose residue present at non-reducing end of glycolipids.

Supported by FAPESP, FINEP and CNPq.

S26. CARBOHYDRATES AND CELL ADHESION II: SELECTIN-DEPENDENT CELL ADHESION

Chairs: Jim Paulson, Carl Gahmberg

S26. 4.00pm

Endothelial Sialyl Le^a and Sialyl Le^x as a Target of L-Selectin Dependent Lymphocyte Adhesion, and Synthetic Sialyl Le^x Glycans as L-Selectin Inhibitors

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Acute cardiac graft rejection is characterized by a heavy lymphocyte infiltration. Here we demonstrate that lymphocytes adhere to endothelium of rejecting cardiac grafts, but not to endothelium of syngeneic grafts or normal hearts analysed with the *in vitro* Stamper–Woodruff binding assay. Concomitantly with the enhanced lymphocyte adhesion, the cardiac endothelium begins *de novo* to express sialyl Le^a and sialyl Le^x epitopes, which have been shown to be sequences of L-selectin counterreceptors. The endothelium of allografts, but not that of syngeneic grafts or normal controls, also reacted with the L-selectin-IgG fusion protein, giving further proof of inducible L-selectin counterreceptors. The lymphocyte adhesion to endothelium could be significantly decreased either by treating the lymphocytes with anti-L-selectin antibody, or by treating the tissue sections with sialidase or anti-sialyl Le^a or anti-sialyl Le^x mAbs. Finally, we enzymatically synthesized several members of the sialyl Le^x-family oligosaccharides and analysed their ability to block lymphocyte adhesion to cardiac endothelium. Monovalent sialyl Le^x (a tetramer), divalent sialyl Le^x (a decamer) and tetravalent sialyl Le^x (a 22-mer) could all significantly reduce lymphocyte binding, but the inhibition by tetravalent sialyl Le^x-glycan was clearly superior to other members of the sLex family. Sialyl lactosamines were used as controls, they lacked fucose but were otherwise similar to the members of sialyl Le^x family and had no effect on lymphocyte binding. These results suggest that L-selectin behaves as a 'functional oligomer' on lymphocyte surfaces.

S26. 4.30pm

Sulfated Carbohydrate Ligands For L-Selectin

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L-Selectin, a member of the selectin family of adhesion molecules, mediates the carbohydrate-dependent attachment of circulating leukocytes to endothelial cells in several models of acute and chronic inflammation. The role of L-selectin in inflammatory disease has stimulated a tremendous amount of interest in the nature of its carbohydrate ligands, and in the design of carbohydrate-based inhibitors. We have recently identified the novel carbohydrate structures 6′-sulfo- and 6-sulfo sialyl Lewis x on the biological L-selectin ligand, GlyCAM-1. Sulfation is a key recognition component in these oligosaccharides, and provides an important lead in the design of small molecules that disrupt L-selectin-mediated adhesion. Here we present the design and construction of simple sulfated disaccharides related to the 6′-sulfo sialyl Lewis x structure. These compounds present multiple sulfate esters at key positions on the core disaccharide lactose. In order to assess their ability to block L-selectin-mediated adhesion, we have developed an inhibition ELISA based on the binding of L-selectin to GlyCAM-1. The positions of sulfation on a disaccharide core are central in dictating L-selectin binding activity, providing a platform from which L-selectin-specific antagonists can be developed.

S26. 4.45pm

Disaccharide Uptake and Priming in Animal Cells: Inhibition of Silyl Lewis x by Acetylated Galβ1-4GlcNAcβ-O-naphthalenemethanol

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Inhibitors of glycosylation provide a tool for studying the biology of glycoconjugates. One class of inhibitors consists of glycosides that block glycoconjugate synthesis by acting as primers of free oligosaccharide chains. A typical primer contains one sugar linked to a hydrophobic aglycone. In this paper we describe a way to use disaccharide as primers. Chinese hamster ovary cells readily take up glycosides containing a pentose linked to naphthol, but they take up hexosides less efficiently and disaccharides not at all. To circumvent this problem, analogues of Xylβ1-6Galβ-*O*-2-naphthol were tested as primers of glycosaminoglycan chains. The unmodified disaccharide did not prime, but methylated derivatives had activity in the order Xylβ1-6Gal$(Me)_3$β-*O*-2-naphthol > Xylβ1-6Gal$(Me)_2$β-*O*-2-naphthol ≫ Xylβ1-6Gal(Me)β-*O*-2-naphthol. Acetylated Xylβ1-6Galβ-*O*-2-naphthol also primed glycosaminoglycan efficiently, suggesting that the terminal xylose residue was exposed by removing the acetyl groups. The general utility of acetyl groups to create disaccharide primers was shown by the priming of oligosaccharides on peracetylated Galβ1-4GlcNAcβ-*O*-naphthalenemethanol. This disaccharide inhibited silyl Lewis x expression on HL-60 cells and thereby blocked adhesion to activated èndothelial cells.

S26. 5.10pm

Constitutive Endogenous Lectins of Endothelial Cells

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Endogenous lectins are constitutively expressed on endothelial cell surfaces; they are highly sensitive to environmental conditions. Endothelial cells from post capillary venules of peripheral lymph nodes (PLN) have been immortalized: a cell line HECa10 [1] has been selected with the aim of studing the molecular mechanisms of cell recognitions. These cells express a membrane lectin which is specific for α-L-fucosides. Its surface expression is increased by cytokines, such as IL7 and by conditioned medium from PLN activated lymphoid cells.

The α-L-fucose specific lectin is Ca^{2+} dependent and differs from P- and E-selectins, it was isolated by affinity chromatography on α-L-fucoside immobilized on Sepharose, as a 31 000 M_r protein.

The isolated α-L-fucose specific lectin binds to murine EL4-IL2 T lymphoma cells, which home into peripheral lymph nodes and adhere to a monolayer of HECa10 cells, but not to EL4 T lymphoma cells which do not home to lymph nodes and do not bind to HECa10 cells. The lectin mediates adhesion through binding to glycoconjugates which contain fucose residues. Furthermore, binding of fucoside-containing glycoproteins to HECa10 cells does not modify MECA79, MECA367 addressins constitutive expressions, although it induces the activation dependent expression of E- and P-selectins and up regulates the fucose specific lectin on the membrane.

1. Bizouarne *et al.* (1993) *Biol Cell* **79**: 209–8.

S26. 5.25pm

Molecular Species of Sialyltransferase Involved in Enhanced Expression of Sialyl Lewis a, the Selectin Ligand, in Human Colonic Cancer Tissues

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Sialyl Lewis a, as well as sialyl Lewis x, serves as a ligand for selectins in the adhesion of cancer cells to vascular endothelial cells, and is thought to be implicated in cancer metastasis. Several clinical statistics indicate that the patients with colon cancer have significantly reduced survival rates when the cancer cells strongly express sialyl Lewis a. We studied mRNA expression of glycosyltransferase isozymes, which could be the candidate enzymes in the enhanced synthesis of sialyl Lewis a antigen in colon cancers, and compared their expression with that in the non-malignant colonic epithelial cells prepared from the same patient. Colon cancer tissues expressed several α1 → 3/4 fucosyltransferases (Fuc-T III, IV and VI) and α2 → 3 sialyltransferases (Hst-4 and HST-30, a human counterpart of the rat ST-30). The expression of Fuc-T III, VI was quite variable and not correlated with the expression of sialyl Lewis a antigen in cancer tissues. The expression of Hst-4 was significantly decreased in most cancer tissues. The most conspicuous finding was that the expression of ST-30 was remarkably increased in most cancer tissues when compared to non-malignant colonic epithelial cells. Since the action of the ST-30 on the sialylation of type 1 chain precursor had not been documented, we transfected the HST-30 cDNA to Cos-7 cells, and could detect a significant synthetic activity of the sialyl Lewis a precursor in homogenates of the transfected cells.

S26. 5.50pm

Induction of TNF-α Release from Human Monocytes through the Selectin-Carbohydrate Interaction

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The selectin-carbohydrate interaction has been shown to regulate leukocyte trafficking. We have previously reported that the interaction also modulated leukocyte function including superoxide anion production. In the present study, we investigated effects of P-selectin on the release of tumour necrosis factor-α (TNF-α) from monocytes. TNF-α is known to play essential roles in the progression of inflammatory processes and the immunological regulation of tumour growth in tissues. When monocytes were cultured in a plate which had been coated with P-selectin, TNF-α release to the medium was detected after the culture for 4 h. The maximal response was observed when P-selectin was coated on a plate at a concentration of 0.3 $\mu g\,ml^{-1}$. Anti-sialyl Le^x antibody (KM-93) also induced the release of TNF-α in a dose-dependent manner. These results

suggest that the cell surface lectin-carbohydrate interaction regulated the leukocyte differentiation and effector functions.

S26. 6.05pm

Peptides Inhibit Selectin-Mediated Cell Adhesion *In Vitro*, and Neutrophil Influx into Inflammatory Sites *In Vivo*

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[2]*Department of Chemistry and Biochemistry, San Francisco State University, San Francisco, CA 94132, USA.*

The selectins are cell adhesion molecules whose carbohydrate-binding domain (C-type lectin) is thought to be involved in leukocyte adhesion to activated vascular endothelium in the inflammatory process. A series of peptides, based on a conserved region (48YYWIGIRK55-NH2) of the lectin domain of E-, L- and P-selectins, were anlaysed for their ability to block selectin-mediated cell adhesion *in vitro*, and neutrophil infiltration into sites of inflammation *in vivo*. The peptides inhibited the adhesion of myeloid cells to recombinant forms of E- and P-selectins. The adhesion of myeloid cells to human endothelial cells, stimulated to express E-selectin, was also inhibited by the peptides. Finally, the peptides blocked the adhesion of lymphocytes, expressing L-selectin, to high endothelial venules in lymph nodes which contain the ligand for L-selectin. A clear structure/activity relationship was established when peptides of different amino acid chain lengths were tested in these assays. Peptides lacking tyrosine residues (e.g. WIGIR-NH2) at their amino terminus were poor inhibitors of selectin-mediated cell adhesion *in vitro*. The peptides that were found to be inhibitors of cell adhesion *in vitro* were also found to inhibit (up to 70%) neutrophil infiltration into sites of inflammation in a thioglycollate induced peritonitis mouse model system. They also significantly reduced (>50%) the migration of neutrophils into cytokine treated skin. These results strongly suggest that compounds based on these tyrosine-containing, selectin-derived peptides could be used as anti-inflammatory therapeutic agents.

S26 POSTERS

S26

Glycolipids May Be Endogenous Neutrophil E-Selectin Ligands

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Selectins are C-type lectins involved in leukocyte adhesion. The endogenous human neutrophil ligand(s) for E-selectin has not been definitively characterized. Neutrophil adhesion to immobilized E-selectin was eliminated by neuraminidase pre-treatment of the neutrophils, but not by their pre-treatment with proteases including *O*-sialoglycoprotease [1, 2]. In contrast, P-selectin-mediated neutrophil adhesion was protease as well as neuraminidase sensitive.

Using COS cells transfected with plasmids expressing selectins, we studied the ability of endogenous lipids to support specific cell adhesion. When immobilized on plastic microwells, polar lipid extracts from human neutrophils supported adhesion of E-selectin but not P-selectin expressing COS cells. E-selectin-mediated adhesion was abrogated by pretreatment of the adsorbed extract with neuraminidase but was unaffected by treatment with trypsin. Partitioning of the polar lipid extract to separate gangliosides from phospholipids and neutral lipids resulted in E-selectin-mediated COS cell adhesion to the adsorbed ganglioside-enriched fractions. These data support the hypothesis that an endogenous ganglioside specifically supports E-selectin mediated cell adhesion.

Supported by NIH grants HL14010 and GM07626.

1. Bochner *et al*. (1994) *J Immunol* **152**: 744.
2. Steininger *et al*. (1992) *Biochem Biophys Res Commun* **188**: 760.

S26

Enzymes For The Synthesis Of Nucleotide Sugars

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The variety of sugars appearing in polysaccharides, glycoproteins, glycolipids and other glycoconjugates reflects the diversity of pathways including also the biosynthesis of the activated sugar substrates for glycosyltransferase reactions. The aim of our work is to provide enzymes for the synthesis of nucleotide (deoxy)sugars, and to combine them with glycosyltransferases in order to synthesize relevant glycoconjugates.

The readily reversible reaction of the plant glycosyltransferase sucrose synthase was used for the synthesis of at least five nucleoside diphosphate sugars (NDP-glucose) by cleaving sucrose with nucleoside diphosphates. In combination with kinases and/or dTDP-glucose 4,6 dehydratase the 'bulk' syntheses (> 100 mg scale) of NDP-glucose and/or NDP-4-keto-6-deoxyglucose started from the corresponding nucleoside mono- or diphosphates. A new LacNAc synthesis cycle including only three enzymes was established with *in situ* regeneration of UDP-glucose by sucrose synthase. After optimization of the integrated enzyme reactions 600 mg LacNAc could be synthesized with 1.25 U β1-4 galactosyltransferase.

To extend the spectrum of enzymes for the synthesis of nucleotide sugars we have isolated and/or cloned pyrophosphorylases (UDP-Glc PP and GDP-Man PP) and checked their substrate spectrum with a new nucleotidyl transferase substrate screening assay (NUSSA). The continuous spectrophotometric assay allows the simultaneous check of different sugar-1-phosphates and nucleoside triphosphates in a microtitre plate.

S26

Myeloglycan, a Series of E-Selectin-Binding Polylactosaminolipids (PLAs) Found in Normal Human Leukocytes and Myelocytic Leukaemia HL60 Cells

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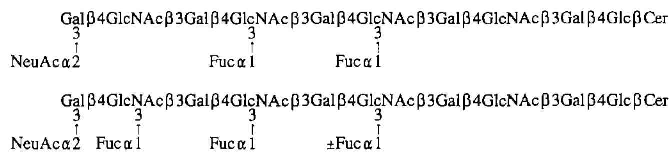
Sialosyl-Le^x (SLe^x) is assumed to be the binding epitope of E- and P-selectin [1, 2]. These and other studies were all based on

inhibition by or adhesion to some *suspected* structure. We studied the E-selectin-binding carbohydrates extracted from a large quantity ($\geq$ 1100 ml) of HL60 cells or 100 ml of neutrophils. Monosialo-ganglioside fractions were selected based on binding to ^{32}P-labelled Chinese hamster ovary cells which permanently express E-selectin. PLA structures were analysed by ^{1}H-NMR, antibody-binding assay, and ES-MS/CID. Hitherto-known SLe^x epitopes such as IV^{3}NeuAcIII^{3}FucnLc$_4$Cer, VI^{3}NeuAcV^{3}FucnLc$_6$Cer, and VI^{3}NeuAcV^{3}FucIII^{3}FucnLc$_6$Cer were all absent. Long-chain PLAs with internally monofucosylated structure, i.e. X^{3}NeuAcVII^{3}FuncLc$_{10}$Cer or X^{3}NeuAcV^{3}FucnLc$_{10}$Cer, were present but did not bind to E-selectin. Fractions 13-1 and 13-2, which bound to E-selectin, contained a common major component which was identified as X^{3}NeuAcVII^{3}FucV^{3}FuncLc$_{10}$Cer (structure A below), and a minor component, X^{3}NeuAcIX^{3}FucVII^{3}FucV^3 ± FucnLc$_{10}$Cer (structure B). These terminally-sialylated, internally polyfucosylated PLAs are termed 'myeloglycan', and are suggested to be physiological ligands of E-selectin.



1. Phillips ML, Nudelman ED *et al.* (1990) *Science* **250**: 1130.
2. Polley MJ, Phillips ML *et al.* (1991) *PNAS* **88**: 6224.

S26

Mucins Containing the Extracellular Parts of MUC1 and CD43 as Apoproteins Carry Sialyl-Lewis a/x Epitopes, are Released from Tumour Cells and Can Inhibit Leukocyte Adhesion

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The colorectal carcinoma cell line COLO 205 produces two mucins that both carry sialyl-Lewis a and to a minor degree sialyl-Lewis x epitopes. The larger of these mucins has been shown to have the MUC1 apoprotein as protein core and the smaller have recently been shown to have the CD43 (leukosialin) core. The CD43 mucin was shown by Edman degradation, Northern blots, and precipitation of precursor apoprotein by antibodies against deglycosylated small mucin from COLO 205 cells and a cytoplasmic CD43 synthetic peptide.

The mucins in the sera of two patients with advanced colorectal cancer were shown to contain large amounts of sialyl-Lewis a and x expressing mucins showing reactivity with MUC1 apoprotein antibodies. This MUC1 fraction was shown to be devoid of its cytoplasmic domain as was also the MUC1 mucin from the COLO 205 cells. The two patients also had a smaller sized mucin fraction expressing sialyl-Lewis a epitopes, but almost no sialyl-Lewis x epitopes.

These secreted mucins are potential inhibitory molecules for the selectin-dependent leukocyte-endothelial cell interactions. This has been tested by analysing the adhesion of HL-60 leukaemia cells to E-selectin transfected COS-1 cells and IL-1β stimulated endothelial cells. The crude sera of the two advanced cancer patients inhibited the leukocyte adhesion as did the purified mucin fractions. The MUC1 mucins from both the COLO 205 cells and patients had about a 10-fold higher inhibitory effect (same sialyl-Lewis a level) than the CD43 mucin from COLO 205 and the smaller mucins from the patient. Secretion of this type of mucins could help the tumour cells escape the immune surveillance and in advanced cancer patients contribute to their immunodepressed condition.

S26

Synthesis of O-Naphthalenemethanol Glycosides – Potential Inhibitors of Glycoconjugate Biosynthesis

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2-Acetamido-1,4,6-tri-*O*-acetyl-3-deoxy-3-fluro-α-D-glucopyranose has been demonstrated to serve as an inhibitor for the biosynthesis of cell adhesion glycoproteins [1]. However, Esko *et al.* [2] have now shown that more easily accessible O-naphthalenemethanol derivatives can likewise inhibit glycoconjugate biosynthesis. In a continuing effort to provide more useful moieties for employment in inhibition studies, we have initiated a program on the synthesis of naphthalenemethanol derivatives of β-D-GlcNAc and α-D-Gal linked oligosaccharides. Our strategy in the creation of inhibitors is defining the specific acceptor substrate for the enzyme then chemically synthesizing an analogue having a masking group, i.e. *O*-methyl, positioned on the hydroxy group that would normally serve as the point of attachment for the transferred glycosyl unit. On the basis of this rationale, we have already accomplished the synthesis of 3-*O*-MeGlcNAc-β-D-naphthalenemethanol (I), 6-*O*-MeGlcNAcβ-naphthalenemethanol (II), Galβ(1 $\rightarrow$ 3)GlcNAc-β-*O*-naphthalenemethanol (III), 3-*O*-MeGalβ-(1 $\rightarrow$ 3)-GlcNAc-β-*O*-naphthalenemethanol (IV), Galβ(1 $\rightarrow$ 3)6-*O*-MeGlcNAc-β-*O*-naphthalenemethanol (V), Galβ(1 $\rightarrow$ 4)GlcNAc-β-*O*-naphthalenemethanol (VI), Galβ(1 $\rightarrow$ 4)3-*O*-MeGlcNAc-β-*O*-naphthalenemethanol (VII), GlcNAc-β-(1 $\rightarrow$ 6)-2-*O*-MeGalα-*O*-naphthalenemethanol (VIII) and Galβ(1 $\rightarrow$ 4)-GlcNAc-β(1 $\rightarrow$ 6)-2-*O*-MeGalα-*O*-naphthalenemethanol (IX).

Supported by NCI Grant No. CA 35329.

1. (1994) *J Biol Chem* **269**: 22797–803.
2. Esko *et al.* (1994) *J Biol Chem* **269**: 300–7.

S26

Metastasis Model of Human Colon Cancer Cells to Liver-Sialyl-Lewis x Expressing Colon Cancer Cells Adhere to Liver Tissue

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We have previously reported that colon cancer cells metastasized to liver express increased amounts of sialyl-Lewis x (SLeX) antigen compared to their primary lesions. It is well known that SLeX and sialyl-Lewis a antigens are ligands for selectins expressed on the endothelial cells. However, it is controversial whether human liver sinusoidal endothelial cells express selectins or not. In this report we tried to make a model of human colon cancer cell metastasis to liver, which was treated with IL-1β in order to induce selectin on the sinusoidal endothelial cells. Immunohistochemical study revealed that

IL-1β treated liver endothelial cells show weak positive staining with anti-selectin monoclonal antibody. The adhesion of SLeX-high or -low expressing colon cancer cells to the liver tissue was examined by Stamper-Woodruff assay. Significantly high number of SLeX-high colon cancer cells adhered to the IL-1β treated liver tissue compared to SLeX-low colon cancer cells. The adhesion of SLeX-high colon cancer cells was inhibited by pretreatment of tumour cells with anti-SLeX antibody or by pretreatment of liver tissue section with anti-selectin antibodies. It becomes clear that SLeX expressing cancer cells could metastasize to liver via SLeX-selectin interaction.

S26

Selectin Ligands: Synthesis of β-D-Gal-(1 → 4)-6-*O*-Sulfo-GlcNAc-β-(1 → 6)-[-β-D-Gal-(1 → 3)]-GalNAc-α-*O*-Bn as a Precursor Structure for Branched Structures

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GLYCAMI and CD_{34} are two HEV associated ligands of L-selectin. Both ligands are mucin-like glycoproteins containing fucose, sialic acid and sulfated O-linked carbohydrate chains. Our laboratory has initiated a program for the chemical synthesis of oligosaccharides which are likely to be a part of such ligands. Our chemical strategy and rationale for the synthesis of benzyl *O*-(β-D-galactopyranosyl)-(1 → 4)-*O*-(2-acetamido-2-deoxy-6-*O*-sulfo-β-D-glucopyranosyl sodium salt)-(1 → 6)-*O*-[β-D-galactopyranosyl-(1 → 3)]2-acetamido-2-deoxy-α-D-galactopyranoside will be presented. Ethyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1 → 4)-3-*O*-acetyl-6-*O*-chloroacetyl-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside is utilized as an important glycosyl donor. Enlisting enzyme catalysis techniques our title compound can be utilized as a substrate for the preparation of branched chain structures; eg. Galβ1 → 4[Fucα1 → 3]6-*O*-sulfoGlcNacβ1 → 6[Galβ1 → 3]GalNAc-α1-*O*-Bn, NeuAcα2 → 3Galβ1 → 4[Fucα1 → 3]6-*O*-sulfoGlc-Nacβ1 → 6[NeuAcα2 → 3Galβ1,3]GalNAcα-*O*-Bn, and others, which have been reported to comprise the carbohydrate structure from mucin glycoprotein [1].

1. Lamblin *et al*. (1994) *J Biol Chem* **269**: 18794–18813.
Supported by Grant No. CA 35329.

S26

Priming of Leukocytes for Superoxide Anion Release by Cytokines Is Associated with Redistribution of Selectin Counter Ligands on Cell Surfaces

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During the inflammatory process, neutrophils and monocytes adhere to activated platelets and vascular endothelial cells. This adhesion has been shown to lead to the activation of leukocytes to generate extracellular superoxide anion [1]. The signal for this activation is apparently sent through P-selectin on platelets or endothelial cells and sialyl Lewis x carbohydrate structure on leukocytes. Immobilized recombinant P-selectin induced superoxide anion release from leukocytes. However, recombinant soluble P-selectin is incapable of inducing superoxide anion, unless the cells are primed by pretreatment with interleukin-8, granulocyte colony stimulating factor, or granulocyte-macrophage colony stimulating factor. During this priming period, sialyl Lewis x epitopes are shown to redistribute to one end of the cells. The pretreatment of primed leukocytes with cytochalasin B inhibited the redistribution of sialyl Lewis x epitopes and superoxide anion generation. Furthermore, sialyl Lewis x epitopes seemed to be concentrated to the cell surface area attaching to immobilized P-selectin. It is strongly suggested that redistribution and increased local concentration of P-selectin ligands may be a prerequisite for leukocyte activation through P-selectin.

1. Nagata *et al*. (1993) *J Immunol* **151**: 3267–73.

S26

Novel Glycoproteins Inhibiting the Binding of Colorectal Cancer Cells to E-Selectin

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Mucin-type glycoproteins carrying sialyl-Le^a antigens (SL-GP) were isolated from ascites fluid from a patient with colorectal cancer, by immunoaffinity chromatography. Their chemical properties including the binding to E-selectin were investigated. The SL-GP showed a typical amino acid composition of a mucin, in which Ser, Thr and Pro together accounted for greater than 50% of the total amino acid residues and the composition was closely related to MUC2 protein. Carbohydrate moieties of the SL-GP were composed of SA, Fuc, Gal, GalNAc, GlcNAc and Man, suggesting the presence of both O- and N-glycans. A large amount of carbohydrates (~80%) were present in the SL-GP, most of which were O-glycans comprising long chains including sialyl-Le^a antigens and the low molecular species including incompletely synthesized carbohydrate chains and their sialylated counterparts, such as the Tn and sialyl-Tn antigens. The O-glycans carrying the sialyl-Le^a antigen comprised about 9% of total O-glycosidic chains.

The SL-GP could bind to IL-1β treated HUVEC. It should be noted that the binding of colorectal cancer cells, LS180 to IL-1β treated HUVEC was inhibited by quite a small amount of the SL-GP (200 $ng\,ml^{-1}$). The binding of ^{125}I-labelled SL-GP to HUVEC was Ca-dependent and inhibited by anti-sialyl-Le^a antibody (MSW113) or anti-E-selectin antibody but not by anti-P-selectin antibody.

S26

Site-Directed Enzymatic Fucosylation of a Multivalent Oligosaccharide Acceptor: A Route to the Synthesis of Pure Lex and sLex Structures

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A sequence of enzymatic reactions is described that allows site specific α1,3-fucosylation of the tetrasaccharide Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc at the distal *N*-acetyllactosamine unit. It involves (i) protection of the 'inner' *N*-acetyllactosamine unit at the reducing end by mid chain β1,6-*N*-acetylglucosaminylation; (ii) the site-specific fucosylation step performed

with human milk α1,3-fucosyltransferase(s), which do not act at the branching *N*-acetyllactosamine units [1]; and (iii) removal of the protecting group with β-*N*-acetylhexosaminidase. This reaction sequence leading to a pure Lewis x pentasaccharide Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4GlcNAc is also applicable to synthesis of pure sialyl Lewis x oligosaccharides, free of VIM-2 type isomers or difucosylglycans.

We thank Dr C. Costello, Boston University School of Medicine, Mass Spectrometry Resource, Boston, MA, USA, for the MALDI mass spectra. Grants from the University of Helsinki and from the Academy of Finland are acknowledged.

1. Niemelä R *et al*. (1995) *Glycoconjugate J*, in press.

S26

Role of Glycosylation in Degradation and Turn-over of E-selectin

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E-selectin is a cytokine-inducible, membrane glycoprotein capable of mediating adhesion of leukocytes to endothelial cells. It is highly glycosylated, containing 11 sites for N-linked glycosylation.

N-glycosylation of E-selectin was analysed by endo-glycosidase treatment. SDS-PAGE analysis of immunoprecipitated E-selectin from human umbilical vein endothelial cells (HUVEC) showed that E-selectin was completely resistant to Endoglycosidase H, but sensitive to peptide N-glycanase F digestion. This suggested that all N-linked oligosaccharide chains were of the complex type.

The role of N-linked glycosylation in surface expression and secretion of E-selectin was studied using interleukin-1 stimulated HUVEC, cultured in the presence of the soluble glycosylation inhibitors tunicamycin or castanospermine. Cell surface expression was analysed by indirect flow cytometry. *N*-Glycosylation was blocked by tunicamycin, and resulted in a significantly reduced surface expression of E-selectin, wheres castanospermine only marginally reduced E-selectin expression. The deglycosylated forms of E-selectin were also found to be fully capable of mediating adhesion of HT-29 cells *in vitro*.

The role of N-linked glycosylation in degradation and turnover rates of E-selectin was studied using pulse-chase experiments. Degradation of E-selectin was further studied by culturing HUVEC in the presence of inhibitors of lysosomal function.

In conclusion, these studies show that E-selectin is heavily glycosylated with complex type N-linked oligosaccharides and that *N*-glycosylation is important for appropriate expression of E-selectin on human endothelial cells. Furthermore, deglycosylated E-selectin was found to be more rapidly degraded after internalization compared to the fully glycosylated protein.

S26

Synthetic Oligovalent Sialyl Lewis x Glycans Are High-Affinity Inhibitors of L-Selectin-Mediated Lymphocyte Binding to Endothelium

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Kidney transplant rejection is an inflammatory process characterized by lymphocyte infiltration. Our ealier observations have pointed out that peritubular capillary endothelium (PCTE), which acquires morphological features common to lymph node high endothelium and begins to express sialyl Lewis x (sLe^x) *de novo*, is the site of L-selectin-dependent lymphocyte entry into the rejecting renal allograft. Inhibiting the lymphocyte-endothelial interaction by utilizing oligosaccharide ligands of L-selectin offers an attractive possibility to prevent inflammation and rejection. The soluble low antigenic ligands compete for L-selectin thus preventing the binding of the endothelial oligosaccharide-containing counter-receptors. Here, we report data on enzyme-assisted synthesis of *N*-acetyllactosamine-based tetra-, deca- and docosasaccharides carrying one, two or four distally located sialyl Lewis x [NeuNAcα2-3Galβ1-4(Fucα1-3)GlcNAc] residues, or sialyl-α2,3-*N*-acetyllactosamine residues, repectively. The structures of the oligosaccharides were verified by utilizing chromatographic experiments, enzymatic degradations, NMR and mass spectrometry. When tested for their ability to inhibit L-selectin-dependent lymphocyte-endothelial interaction during rat kidney transplant rejection, all sLex-containing constructs were effective inhibitors in the Stamper-Woodruff binding assays; the analogues lacking fucose showed no inhibitory potency. The tetravalent sLex glycan was clearly a more potent adhesion inhibitor than the divalent sLex construct, while the latter was more potent than the monovalent sLex saccharide. This suggests that the tetravalent sLex glycan may adhere to more than one binding site belonging to several L-selectin monomers on the cell surface.

S26

Identification of a 120 kDa Glycoprotein as a Ligand for L-Selectin in Porcine Peripheral Lymph Nodes

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Chronic immunoinflammatory diseases such as inflammatory bowel disease and rheumatoid arthritis are characterized by the massive migration of lymphocytes at the sites of inflammation. The trafficking of lymphocytes from the blood and into the lymphoid organs is controlled, in part, by a primary adhesive interaction between L-selectin and peripheral addressin (PNAd). Two sulfated, sialylated and fucosylated glycoproteins of 50 kDa (sgp 50, GlyCAM 1) and 90 kDa (sgp 90; CD34) have been identified from mouse peripheral lymph nodes as ligands for L-selectin. We have identified two glycoproteins of 80–90 and 120 kDa in porcine peripheral lymph nodes that are sulfated, sialylated and fucosylated. Both of these glycoproteins are recognized by a monoclonal anti-PNAd antibody (MECA-79). We have purified the 120 kDa glycoprotein and demonstrated that it binds to a human L-selectin-Fc chimera in a specific and calcium-dependent manner. Recent data suggest that the recognition epitope on PNAd is related to the sulfated form of sialyl-Lewis X blood group antigen. Utilizing chlorate as a metabolic inhibitor of sulfation in organ culture, we have also demonstrated that the sulfation of the 120 kDa protein appears to be absolutely essential for its interaction with L-selectin as well as with MECA-79. The sulfation that confers the L-selectin binding activity appears to be on O-linked glycans of the 120 kDa protein.

S26

Sulfated Glycolipids as Potential Ligands for L- and P-selectins and their Role in Inflammation in Kidney and Lung

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Selectins are adhesion-promoting molecules that facilitate, via their lectin binding sites, adhesive interactions between leukocytes and activated endothelial cells. We demonstrate here that sulfated glycolipids, especially sulfatide, reacts with L- and P-selectins and has powerful anti-inflammatory effects on the L- and/or P-selectin dependent rat kidney and lung injury.

L-selectin ligand in rat normal kidney stained with L-selectin-IgG chimera is located in the epithelial cells of the distal tubes. This location was very similar to that of sulfatide which was stained with anti-sulfatide MoAb. Sulfatide ligand was redistributed into the interstitium of the kidney after ureteral obstruction, a major model for human obstructive nephropathy. This sulfatide redistribution was accompanied with L-selectin dependent infiltration of the numerous mononuclear cells from peritubular capillaries which closely contact with distal tubules in the kidney, and was strongly prevented by the i.v. administration of sulfatide.

It was also demonstrated that L- and P-selectin dependent lung inflammation induced by cobra venom factor, a model of human adult respiratory distress syndrome (ARDS) was strongly prevented by the i.v. perfusion of sulfatide or related sufated sugar ligands.

S26

Activation of Lymphocytes via L-Selectin: Induction of Homotypic Adhesion and Altered Binding Capacity to HEV

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L-selectin (CD62L) is a member of the carbohydrate-binding family of selectin cell surface molecules and is especially involved in the initial and transient attachment of lymphocytes to high endothelial venules (HEV) in lymphoid organs. A role for sugar moieties in lymphocyte-HEV interactions has been inferred from the inhibitory effects of several carbohydrates on this interaction *in vitro*. Recently an L-selectin dependent, rapidly induced homotypic adhesion among both normal lymphocytes and lymphoblastoid cells has been described.

Here we have used this aggregation model to study the role of carbohydrates in the activation of lymphocytes via L-selectin. Various anti-L-selectin antibodies and carbohydrates were tested upon their ability to induce homotypic adhesion in both human and murine lymphocytes expressing L-selectin. Furthermore, aggregated cells were tested with respect to their binding capacity to PLN-HEV. We have shown that the interaction of the carbohydrate recognition domain of L-selectin with fucoidan, or with the monoclonal antibodies Mel-14 and LAM1/3, results in a calcium-independent and energy-dependent homotypic aggregation. Furthermore, aggregation via L-selectin does not involve an increased cytosolic calcium concentration. However, specific tyrosine-phosphorylation of several proteins could be observed. Interestingly, we found that the L-selectin triggered cells show an altered binding capacity to PLN-HEV.

These results suggest that triggering of aggregation via L-selectin by fucoidan might be similar in many aspects to the initial *in vivo* activation during lymphocyte binding to HEV.

S26

Induction of Endothelial Sialyl Lewis^a and Sialyl Lewis^x During Cardiac Transplant Rejection: Superior Capacity of a Tetravalent sLex-Oligo-saccharide in Inhibiting L-Selectin-Dependent Lymphocyte Adhesion

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Acute organ transplant rejection is characterized by a heavy lymphocyte infiltration. In this study we demonstrate that lymphocytes adhere to endothelium of rejecting cardiac transplants, but not to endothelium of syngeneic grafts or normal hearts analysed with the *in vitro* Stamper-Woodruff binding assay. Concomitantly with the enhanced lymphocyte adhesion, the cardiac endothelium begins to *de novo* express sialyl Lewis a and sialyl Lewis x (sLea and sLex) epitopes, which have been shown to be sequences of L-selectin counter-receptors. The endothelium of allografts, but not that of syngeneic grafts or normal controls, also reacted with the L-selectin-IgG fusion protein. The lymphocyte adhesion to endothelium can be significantly decreased either by treating the lymphocytes with anti-L-selectin antibody HRL-1, or by treating the tissue sections with sialidase or anti-sLea or anti-sLex mAbs. The lymphocyte adhesion can be blocked to background levels when anti-L-selectin and anti-oligosaccharide mAbs are applied together. Finally, we analysed the ability of several members of the sLex-family to block lymphocyte adhesion to cardiac endothelium. Monovalent sLex (tetramer), divalent sLex (decamer) and tetravalent sLex (22-mer) could all significantly reduce lymphocyte binding, but the tetravalent sLex-construct was clearly superior to other members of the sLex family.

S27. TRANSMEMBRANE SIGNALLING CONTROL BY GLYCOSPHINGOLIPIDS

Chairs: Subroto Chatterjee, Shizuo Handa

S27. 400pm

Overview: Glycosphingolipids and Signalling

S27. 4.05pm

Lactosylceramide (LacCer) is a Transmembrane Signal for Cell Proliferation

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We have previously shown that LacCer serves as a mitogenic agent in the proliferation of human aortic smooth muscle cells (SMC), a hallmark in the "pathogenesis of atherosclerosis" [1]. In agreement with this notion, we found that LacCer accumulates in large amounts in the intimal plaques of patients who died of atherosclerosis. Here, we sought to investigate LacCer mediated signalling events that may contribute to the proliferation of SMC. We found that LacCer exerts a time (10–15 min) and concentration (10 μM) dependent specific stimulation (three to five-fold compared to control) in the expression of p44 mitogen activated protein kinase (MAPK) in these cells. Preincubation of cells with cytochalasin-D, a potent inhibitor of microfilament assembly but not cycloheximide (100 $\mu g\, ml^{-1}$), markedly inhibited the phosphorylation of p44 MAPK. LacCer did not alter the mRNA levels of MAPK-phosphatase in these cells.

In summary, LacCer mediates signal transduction events such as the activation of (phosphorylation) of p44 MAPK. That in turn, contributes/activates certain nuclear factors responsible for the proliferation of aortic smooth muscle cells.

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1. Chatterjee S (1991) *BBRC* **181**: 554–61.

S27. 4.30pm

Ganglioside Binding with the Specific Enzyme Domain Resulting in the Modulation of the Calmodulin-Dependent Enzyme

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In order to further confirm the notion that gangliosides modulate calmodulin-dependent enzyme activity through binding to a specific enzyme domain as well as to calmodulin, we examined the interaction of gangliosides with synthetic peptides of several calmodulin-dependent enzymes. Synthetic peptides corresponding to the calmodulin like binding site (CLBS) as well as the calmodulin-binding site (CBS) of the enzyme prevented gangliosides from inhibiting the enzyme activity. These results indicate that direct activation of calmodulin-dependent enzymes occurs through the binding of gangliosides to the CBS at lower ganglioside concentrations and that gangliosides directly inhibit the enzyme activity through futher binding to the CLBS at higher concentrations.

Moreover, using gel chromatography, we have not only confirmed the binding of gangliosides to the peptide which had been expected from the enzyme kinetics but also found the presence of a positive binding between the peptide and gangliosides which had been thought to be negative.

These interactions between gangliosides and the enzyme explain the bidirectional modulation of enzyme activity by gangliosides.

S27. 4.55pm

Plasmalopsychosine of Human Brain Mimics Nerve Growth Factor Activity by Activating its Receptor Kinase and Mitogen-Activated Protein Kinase, Inducing Differentiation in PC12 Cells

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Plasmalopsychosine, a characteristic fatty aldehyde conjugate of β-galactosyl-sphingosine (psychosine) found in brain white matter [1], has now been found to mimic the effects of nerve growth factor (NGF). It strongly enhances p140^{trk} phosphorylation and mitogen-activated protein kinase (MAPK) activity, and as a consequence induces neurite outgrowth in PC12 cells. Plasmalopsychosine's effect on neurite outgrowth was stronger than that of NGF, and its prolonged activation of MAPK was similar to that of NGF. Tyrosine kinase inhibitor K-252a and staurosporine, known to inhibit the neurotrophic effect of NGF, also inhibited these effects of plasmalopsychosine, suggesting that plasmalopsychosine and NGF shares a common signalling cascade. Plasmalopsychosine may play an important role in development and maintenance of the vertebrate nervous system.

Supported by NCI-JFCR fellowship (to C.S.) and NCI grant CA 42505 (to S.H.).

1. Nudelman *et al.* (1992) *J Biol Chem* **267**: 11007–16.

S27. 5.10pm

Glucosylceramides Stimulate Hyperproliferation of Young and Aged Murine Epidermis

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Whereas numerous studies have focused on ceramides as growth inhibitors, GlcCer may act oppositely, that is, as a mitogen. In the present studies we determined whether enhancement of GlcCer content stimulates epidermal mitogenesis, examining the response of both young (<2 months) and older (>24 months) hairless mouse epidermis to alterations in endogenous and/or exogenous GlcCer. In young animals; 1) topical conduritol B-epoxide (CBE), an inhibitor of GlcCer'ase, increased epidermal GlcCer levels, an alteration localized largely to the basal proliferative cell layer; 2) CBE also stimulated epidermal proliferation (1.5-fold elevation in [^{3}H]-

thymidine incorporation; $p < 0.001$), localized autoradiographically again to the basal layer, and resulting in epidermal hyperplasia; and 3) both topical and intracutaneous administration of GlcCer also stimulated epidermal DNA synthesis. In aged animals (>24 months), where baseline [^{3}H]-thymidine incorporation was diminished vs. young animals, topical CBE stimulated epidermal DNA synthesis (up to 1.9-fold). Non-specific irritant effects were excluded because: 1) chemically-related, less effective GlcCer'ase inhibitors and other glycosphingolipids did not increase epidermal proliferation; and 2) coadministration of a potent topical corticosteroid with CBE did not block the mitogenic response. These results provide evidence that GlcCer has direct mitogenic effects in the epidermis, and that aged epidermis retains sensitivity to GlcCer-induced mitogenesis.

S27. 5.25pm

Differential Effects of GM3 and Leflunomide on EGF Receptor Signal Transduction

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The mitogenic effect of EGF has been shown to depend on the induction of EGF receptor tyrosine specific protein kinase activity. This effect leads through MAP kinase and stimulation of early-immediate gene expression, *c-myc*, *c-jun*, and *c-fos*. Phosphorylation of the EGF receptor can be affected by several molecules, most of which are chemical inhibitors of tyrosine kinase activity. Leflunomide is one of these inhibitors. Ganglioside GM3 is a physiological molecule located in the cell membrane that appears to inhibit EGF-induced autophosphorylation of EGF receptor and kinase activity by preventing activation of the receptor. In order to understand how these two classes of inhibitors can also affect its signal transduction, the effect of GM3 and leflunomide on the activation of MAP kinases and gene expression was examined. Dose dependent inhibition of MAP kinase tyrosine phosphorylation was observed both *in vitro* and in intact cells with GM3 and leflunomide. On the other hand, the effects of these two molecules on EGF induced expression of *c-fos* and *c-myc* were quite different. While GM3 was able to inhibit the expression of both *c-myc* and *c-fos*, leflunomide inhibited *c-fos* but had no effect on *c-myc* expression. These results suggest that although there were similar effects of GM3 and leflunomide on EGF dependent phosphorylation, gene expression was different. Preventing activation of the EGF receptor by GM3 had a more profound effect on gene expression than simple inhibition of the tyrosine specific protein kinase activity.

S27. 5.40pm

Stimulation of Tyrosine Phosphoprotein Expression by Anti-GD_3 MoAb in a TCR/$CD3^+GD_3^-$ Malignant T-Cell Line after Incorporation of Exogenous GD_3

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The ganglioside GD_3 is highly expressed in a subclass of resting human T cells, distinguished by memory cell surface markers CD45R0 and CD29, and these cells proliferate in response to the anti-GD_3 monoclonal antibody R24. To examine early T cell activation signalling events which may be linked to GD_3, TCR-$CD3^+$ Jurkat T-lymphocytic leukaemia (T-ALL) cells deficient for GD_3 were tested for altered tyrosine phosphoproteins in response to R24 after incorporation of exogenous GD_3. GD_3 incubation resulted in uptake and surface expression of the ganglioside in a dose and time-dependent manner. GD_3^+ cells were washed, incubated with R24, and analysed for tyrosine phosphoproteins by anti-phosphotyrosine immunoblot analysis of total lysate proteins separated by SDS-PAGE. Within 1 min of R24 incubation, tyrosine phosphorylation of several proteins, particularly p44 ERK1 kinase, increased, while longer incubation resulted in increased Lck kinase molecular weight from 56 kDa to 60 kDa. Incubation of cells with control IgG3 antibody or incubation of untreated Jurkat cells with R24 resulted in no change in tyrosine phosphoproteins (PYP). Preincubation of cells with tyrosine kinase or tyrosine protein phosphatase inhibitors eliminated R24-induced alterations. The kinetics of altered PYP and its profile induced by R24 were essentially the same as those found after treatment of GD_3^+ cells with anti-CD3 antibody, UCHT-1. In contrast to Jurkat cells, MOLT-4 T-ALL cells which constitutively express GD_3 but not the TCR/CD3 complex were insensitive to R24, either untreated or after incorporation of exogenous GD_3. The combined results indicate that R24-stimulated GD_3 signalling in T cells involves a tyrosine phosphorylation cascade, and suggests further the presence of a GD_3/TCR complex that may be cross-linked and activated by R24.

S27. 5.55pm

Control of DNA Replication by Acidic Glycosphingolipids and Sphingosine

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Exogenously added GM3 induces morphological and functional differentiation together with cell growth inhibition. In the present study, we examined the effect of various glycolipids, ceramide and sphingosine on the DNA polymerase activity. Gangliosides and sulfatide inhibited the DNA polymerase a activity in a dose-dependent manner, while other neutral glycolipids, ceramide and sphingosine did not. The *N*-acetyl residue of the sialic acid moiety is particularly essential for inhibition by both SPG and GM3, because the loss of this residue or substitution with a glycolyl residue completely negated their inhibitory effect. Sphingosine strongly inhibited the activity of primase. Dihydrosphingosine, ceramide, glycosphingolipids and sphingomyelin had no effect on primase activity. The inhibitory effect on DNA replication may be related to the apoptosis caused by the exogenous addition of sphingosine.

S27 POSTERS

S27

Lactosylceramide (LacCer)–Mediated Activation of Mitogen-Activated Protein Kinase

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Previously, our laboratory has shown that LacCer can serve as a mitogenic agent in the proliferation of aortic smooth muscle cells [1]. Here we report a novel aspect of LacCer-mediated signal transduction. We demonstrate that LacCer can activate mitogen-activated protein kinase (p44 MAPK), but not MAPK-phosphatase. Within five minutes of incubation with LacCer there was a three-fold increase in the activity of p44 MAPK.

Incubation of cells with Cer and GlcCer did not significantly stimulate p44 MAPK. Preincubation with tyrphostin and cytochalasin-D, an inhibitor of actin microfilament assembly, markedly inhibited the LacCer mediated stimulation in p44 MAPK. However, cycloheximide did not have any effect on the LacCer mediated activation of p44 MAPK. These results indicate an important role for the cytoskeleton in LacCer mediated p44 MAPK activation and cell proliferation.

In summary, LacCer mediated activation of MAP kinase may have important implications in the signalling processes required for the regulation of cell growth in aortic smooth muscle cells, "a hallmark in the pathogenesis of atherosclerosis".

Supported by 1-P50 HL47212 and RO1-DK-31722.

1. Chatterjee S (1991) *BBRC* **181**: 554–61.

S27

Epidermosides in Human Epidermis and Its Relation to Keratinocyte Differentiation

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Major glycosphingolipids of human epidermis were determined to be glucosylβ1-*N*-(ω-*O*-linoleoyl)-triacontanoyl- and -dotriacontamonoenoyl-eicosasphingenine and glucosylβ1-*N*-(ω-*O*-linoleoyl)triacontanoyl-trihydroxyeicosasphingenine, and we named them epidermosides.

When human keratinocytes were cultured and kept in a less undifferentiated condition, only epidermosides containing sphingenine were detected. In addition, five glucosylceramides and a mixture of glucosylceramides and galactosylceramides were detected but glucosylceramides containing long chain ω-hydroxy fatty acids which are assumed to be immediate precursors of epidermosides were not detected. These results suggest that the synthesis of epidermosides in undifferentiated cultured keratinocytes is different from that in epidermis. Presumably, the synthesis of epidermoside is altered during keratinocyte differentiation.

Finally, to study the biological function of epidermosides, we demonstrated that the effect of a chemical synthesized glycosyl-β1-*N*-(ω-*O*-linoleoyl)-triacontanoyleicosasphingenine on differentiation and proliferation of human keratinocytes. Epidermoside at a concentration of 10 μg ml^{-1} increased the keratin content of the cells 5.6-fold and changed their morphological appearance into a differentiated one, while it did not influence cell proliferation. These results suggest that epidermosides have an ability to enhance keratinocyte differentiation.

S27

Characterization of Gangliosides from Ehrlich Ascites Tumour Cells and their Variants

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Differences in the nature of the gangliosides present in two variants of Ehrlich ascites tumour (EAT) cells, the adherent and non-adherent EAT cells, were studied. Gangliosides were isolated by DEAE Sephadex column chromatography and analysed by high-performance thin-layer chromatography. The non-adherent EAT (na-EAT) cells which grow in suspension in the peritoneal cavity of mice were selected for growth on basement membrane and tissue culture plastic to give the adherent EAT (a-EAT) cells. na-EAT cells contained 1.57 nmol lipid-bound sialic acid per mg protein, and at least 12 different gangliosides, including major gangliosides such as GM3, GM2, GM1, GD3, GD1a and GT1b. On the other hand, the ganglioside pattern of a-EAT cells differed significantly from that of na-EAT cells, both quantitatively and qualitatively. The content of lipid-bound sialic acid in a-EAT cells was only 0.24 nmol per mg of protein, a decrease of six-fold. The gangliosides in a-EAT cells were characterized as GL1a and trisialogangliosides and, significantly, a-EAT cells did not contain monosialogangliosides. Neutral glycolipids were isolated from both cell lines and their patterns compared. In contrast to the ganglioside pattern, the neutral glycolipid patterns from both EAT cells were similar; glucosylceramide and lactosylceramide were the major components. In addition to na- and a-EAT cells, a third variant of EAT cells was developed by passage of a-EAT cells in mice via intraperitoneal injection (c/m EAT cells). After repeated passage of c/m EAT cells in mice, the pattern of gangliosides shifted from a-EAT cells to that of na-EAT cells. Alterations of ganglioside composition may be associated with the growth environment of the murine peritoneal cavity.

This research was supported by a grant from the National Cancer Institute (CA 0-27157).

S27

Effect of Bacterial Glycosphingolipids on Phagocytosis and Phagosome-Lysosome Fusion by PMN *In Vitro*

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Glycosphingolipids play important roles in the eukaryotic cell membrane. However, none of the physiological roles or biological activities of bacterial glycosphinogolipids have been reported. In 1990, genus *Sphingomonas* was established based

on the existence of acidic glycosphingolipids. Although genus *Sphingomonas* is an opportunistic pathogen and the pathogenic mechanism is not known, we found a marked difference in the rate of phagocytosis by human polymorphonuclear leukocyte (PMN) between intact and delipidated *S. paucimobilis*. In this study, we tested the effect of acidic glycosphingolipids on phagocytosis by PMN using glycosphingolipid-coated staphylococci. As a result, acidic glycosphingolipids (GL-1:glucuronosylceramide and GL-4:mannosyl-galactosyl-glucosaminyl-glucuronosyl ceramide) isolated from *S. paucimobilis* showed a marked stimulative activity on both phagocytosis and phagosome-lysosome fusion by PMN, while neutral glucosylceramide, including bacterial glycosphingolipid reduced with $LiAlH_4$, had no effect. Since the promotion of the rate of phagocytosis by GL-1 was significantly inhibited by the addition of D-glucuronic acid, it was suggested that the existence of the free carboxyl group of glucuronic acid may be essentially required for the promoting of phagocytosis by PMN. Sulfatide and gangliosides form mammalian tissues also stimulated (Yamaguchi *et al.*, see next abstract) but sulfatide isolated from *Mycobacterium tuberculosis* was inhibitory. Therefore, the structure-phagocytic promotion activity relationship of acidic glycosphingolipids will be discussed.

S27

Modulation of IL-3 Signalling by IL-3-Associated Ganglioside GD1a in Murine Myelogenous Leukaemia Cell Line NFS60 Cells

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Division of Hemopoiesis, Institute of Hematology, Jichi Medical School, Minamikawachi, Tochigi 329-04, Japan.

We have previously described the IL-3-associated expression of ganglioside GD1a in a murine myelogenous leukaemia cell line NFS60 introduced with the *IL-3* gene, and a key regulatory mechanism of GD1a expression by GM3 synthase in the transfectants. These strongly suggested the possible GD1a involvement in IL-3 signalling. We now report the analyses of the exogenous GD1a effect on IL-3 signalling in IL-3-dependent NFS60 cells. Immunoblot analysis using anti-phosphotyrosine antibody revealed that IL-3 stimulated the tyrosine phosphorylation of 110 and 86 kDa proteins. Strikingly, GD1a at 20 μM concentration stimulated the phosphorylation of the same 110 and 86 kDa proteins in the absence of IL-3. In addition, while the control GT1b diminished the intensiy of the bands in the presence of IL-3, GD1a clearly enhanced the tyrosine phosphorylation. These results, together with the previous reports, strongly suggest that GD1a is involved in the normal receptor mediated IL-3 signalling in murine NFS60 cells.

S27

Marked Stimulatory Effect of Sulfatide and Ganglioside on Phagocytosis and Phagosome-Lysosome Fusion by Human PMN *In Vitro*

S. Yamaguchi[1], Y. Miyazaki[2], S. Oka[1] and I. Yano[1]
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[2] *Department of Food Science and Nutrition, School of Human Life and Environmental Science, Mukogawa Women's University, 6-46 Ikebiraki-cho, Nishinomiya, Hyogo 663, Japan.*

The physiological role of glycosphingolipids has been investigated extensively because of their importance in the cellular differentiation processes. We have recently found that bacterial glycosphingolipids show marked stimulatory effect on phagocytosis and phagosome-lysosome (P-L) fusion in human polymorphonuclear leukocyte (PMN) *in vitro*. From the structural similarity of the acidic glycosphingolipids, we expected such activities in mammalian glycosphingolipids, and examined the effect on adhesion, phagocytosis and P-L fusion of human PMN using staphylococcal cells coated with various glycolipids. As a result, adhesion was promoted with GM3 or GT1b coated staphylococci. Phagocytosis and P-L fusion were dramatically stimulated with sulfatide (cerebroside sulfate) and also with GM2, GM3, GD1a and GT1b. However, none of the neutral glycosphingolipids (asialo GM1 etc.) and GM1 were stimulatory, indicating that an acidic group such as sialic acid or sulfate is necessary. However, not only the existence of acidic group, but also a microenvironment or localization of acidic function in the molecule is also important for such activities. Since staurosporine (0.04–1 μM) (a protein kinase C inhibitor) inhibited ganglioside-induced phagocytosis, therefore protein kinase C may be involved in phagocytosis stimulation.

FRIDAY 25 AUGUST, MORNING

PLENARY LECTURES

S28. 8.00am

The Role of Endogenous Lectins as Chaperones in Glycoprotein Folding

A. Helenius, D. Hebert, U. Tatu, J. Simons, J. Peterson, Wei Chen, B. Foellmer and A. Ora
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Our studies in live tissue culture cells, in yeast and in isolated microsomes focus on the folding, oligomeric assembly and quality control processes that glycoproteins undergo in the ER. We have recently shown that two unique ER chaperones, calnexin (a membrane protein) and calreticulin (a soluble lumenal protein) bind to growing nascent chains and to newly synthesized glycoproteins. Both are lectins with specificity for the monoglucosylated oligosaccharide side chains. They constitute part of a sophisticated machinery which includes glucosidases I and II as signal modifiers, and UDP-glucose: glycoprotein glucosyltransferase as a folding sensor. Using influenza haemagglutinin and VSV G protein as models, we have shown that the interaction between calnexin/calreticulin and their substrate glycoproteins increases the efficiency but

decreases the rate of folding, delays oligomerization, prevents degradation and blocks premature ER to Golgi transport. Nevertheless, the pathway can be dispensable for many glycoproteins and for growth in wt strains owing to redundance of chaperones in the ER. It is necessary when other chaperones, such as kar2p, are compromised.

S29. 8.30am

Gangliosides as Signalling Molecules In Neural Functions

*Y. Nagai**

The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan and Glycobiology Research Group, Frontier Research Program, Institute of Physical and Chemical Research (RIKEN), Saitama, Japan.

Biosynthesis of major brain gangliosides is usually classified into three pathways, a, b and c. The three pathways are developmentally regulated. GD3 and GQ1b in b-series are of particular interest due to their roles in neural differentiation and functions. GD3 ($II^3(NeuAc)_2LacCer$) characterizes neural cells (neuron and glia) in proliferation, and also the growth of some malignant cells. GQ1b ($IV^3(NeuAc)_2II^3(NeuAc)_2Gg$-Ose4Cer) characterizes neuronal cells in differentiation. Exogenous GQ1b induces and promotes neurite outgrowth in human neuroblastoma cells at the order of a few nM concentrations, which is in sharp contrast to other gangliosides which require the order of μM for the activity. The neuritogenic action of GQ1b is mediated through a glycoreceptor at cell surface that specifically recognizes the oligosaccharide structure of GQ1b. This carbohydrate recognition is followed by phosphorylation of particular cell surface protein(s) catalysed by novel, cell surface localized (ecto-type) protein kinase(s) in the presence of extracellular ATP resulting in the promotion of neurite outgrowth. Thus, GQ1b-specific glycoreceptor-mediated signal transduction is carried out by a novel ecto-type protein kinase(s).

Very recently a human cDNA encoding GD3 synthase (CMP-NeuAc: GM3 α2,8-sialyltransferase) the key enzyme for b-series synthesis, was cloned [1, 2, 3]. The cloned enzyme was then found to catalyse synthesis not only of GD3 but also GQ1b from their precursors, GM3 and GT1b, respectively. The transfection of this cDNA into mouse neuroblastoma cells, Neuro 2a, began to synthesize GD3 as well as GQ1b and then spontaneously induced neurite outgrowth but also acetylcholine esterase, giving clear evidence that GQ1b is a physiologically active neuritogenic substance and also that the GD3 synthase gene is the gene responsible for the differentiation of cholinergic neuron [4].

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1. Nara *et al.* (1994) *Proc Natl Acad Sci USA* **91**: 7952–56.
2. Sasaki *et al.* (1994) *J Biol Chem* **269**: 15950–56.
3. Haraguchi *et al.* (1994) *Proc Natl Acad Sci USA* **91**: 10455–59.
4. Kojima *et al.* (1994) *J Biol Chem* **269**: 30451–56.

S30. 9.00am

Cell Surface Glycoconjugates as Attachment Sites for Microbes

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Results are accumulating on the importance of animal cell surface carbohydrates for the specific attachment of microbes and microbial toxins. Also, important crystal structures of complexes of microbial proteins with saccharide have been reported.

The characteristics of selected saccharide epitopes include the known recognition of non-terminal sequences, of importance for the detailed specificity of binding and tropism of infection, and low-affinity binding. Further documentation is gathering on the role of specifically ceramide-linked sequences (non-secreted, cell-bound sites). In human and monkeys the Galα4Gal receptor for uropathogenic *E. coli* is absent from glycoproteins, and the bacterial adhesin recognizing this sequence has been shown by genetical inactivation studies to be decisive for the development of model pyelonephritis in monkeys. The sialic acid-dependent recognition by *Helicobacter pylori* is apparently restricted to polyglycosylceramides. Ceramide-close epitopes may be essential to create membrane proximity for the invasion of the host cell. This may be the explanation for lactosylceramide binding by some microbes, and for the recognition of one-sugar glycolipids by several viruses including HIV and influenza virus.

Heat-labile toxin (LT) of *E. coli*, with the secretion-inducing mechanism in small intestine and binding to GM1 apparently identical with CT properties, was shown to crossbind to Galβ4GlcNAc-R, which may be explained by an Arg in position 13 in the binding site of LT (His in the case of CT). The receptor, earlier shown on rabbit cells for toxin A of *Clostridium difficile*, Galα3Galβ4GlcNAcβ, cannot be expressed on human cells. It may be replaced in the human by crossbinding trisaccharides, including that ending in GalNAcβ3, identified on human red cells.

Supported by CarboMed, Inc.

S28. GALACTINS, MANNOSE-BINDING PROTEINS, AND OTHER ANIMAL LECTINS

Chairs: Sam Barondes, Michel Monsigny

S28. 9.50am

Introduction

S28. 9.55am

Structural and Functional Studies of Galectins

H. Leffler[1], M. Gitt[1], M. Huflejt[1], E. Jordan[1], C. Kayden[1], A. Kanigsberg[2], J. Lobsanov[2], J. Rini[2] and S. Barondes
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[2]*Department of Molecular Genetics and Medicine, University of Toronto, Canada.*

Galectins are animal lectins that have carbohydrate binding domains with characteristic sequence elements and affinity for certain β-galactosides. The eight known mammalian galectins can be subdivided into different types based on their overall organization. For example, galectin-3 has a carbohydrate binding domain linked to another domain, and galectin-4 has two carbohydrate binding domains within one peptide chain. Four solved crystal structures of galectin carbohydrate binding domains show a conserved folding motif and lactose binding site. A second binding site that differs among the galectins may explain variations in the specificity for extended oligosaccharides.

Our functional studies have focused on galectins in cultured epithelial cells. The colon carcinoma T84 cells, which are well established as a model system for the colon crypt epithelium, express both galectin-3 and -4. Although both galectins have properties of cytosolic proteins including lack of a signal peptide, they appear to be externalized by non-classical secretory pathways. In confluent cells, externalized galectin-4 is found at the site of adherens junctions and/or tight junctions, whereas externalized galectin-3 is found at a more basal site along the basolateral membrane. These different localizations suggest that galectin-3 and -4 have different functions, and may interact with different ligands and/or be externalized by different non-classical secretory pathways.

S28. 10.20am

Galectin-3 Expression and Functions in MDCK Cells in 3 Dimensional Culture *In Vitro*

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Galectin-3 is a member of a closely-related family of β-galactoside-binding soluble proteins found in many vertebrate cell types and its developmentally regulated presence in tissues, for example kidney, suggests important biological roles. A polarized expression and secretion of galectin-3 was observed in monolayer-cultured MDCK cells [1]. In this study, the cellular distribution and function of galectin-3 was examined in MDCK cells cultured within a gel matrix. MDCK cells were cultured within Type I collagen or Matrigel to obtain multicellular cysts, and tubule formation was induced in collagen gels with hepatocyte growth factor (HGF). Immunofluorescent staining of these structures using antibodies against galectin-3 and other cell-surface domain markers was carried out either *in situ* or on cryosections and visualized by confocal and conventional epifluorescent microscopy. Our results show that MDCK cells suspended in hydrated collagen gels or Matrigel exhibit differential and polarized galectin-3 expression on the baso-lateral surface domains of cells lining the cysts. The lectin is co-localized with laminin on the basal surface. In tubule-forming cysts, galectin-3 is excluded from the progressing tips of the tubules although its baso-lateral expressions on the cyst body remains. Galectin-3 added exogenously to cultures, as well as antibodies against laminin subunits and integrin β_1 subunit, exerted an inhibitory effect on cyst enlargement of MDCK cells in 3D Matrigel while galectin-3-specific antibodies could promote this process. These results suggest that galectin-3 exerts its effect on MDCK cells in a 3D environment through modulation of both cell-cell and cell-substratum adhesions and the interplay between these adhesions is important in the growth of multicellular aggregates and extensions occurring during normal kidney tubulogenesis.

1. Sato S, Burdett I, Hughes RC (1993) *Exp Cell Res* **207**: 8–18.

S28. 10.35am

Nuclear Galectins are Functionally Redundant in their Activity in PRE-mRNA Splicing

S. F. Dagher[1], S.-Y. Wang[2], J. L. Wang[2] and *R. J. Patterson*[3]
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Galectins are a family of galactose/lactose-specific animal lectins widely distributed in eukaryotic cells. Galectin-1 ($M_r \sim 14$ kDa) and galectin-3 ($M_r \sim 30$ kDa) have been shown to localize to nuclei of mouse 3T3 fibroblasts and HeLa cells and both are found in HeLa splicing extracts. This nuclear localization prompted us to determine whether nuclear galectins are involved in nuclear RNA biogenesis. Addition of galectin-specific saccharides to HeLa nuclear extracts inhibits pre-mRNA splicing whereas addition of saccharides with no affinity for galectins has no effect on RNA processing. To establish that galectins play a direct role in the splicing pathway, galectin depletion and reconstitution experiments were performed.

Depletion of nuclear galectins by adsorption to a lactose-agarose matrix abolished both pre-mRNA splicing and spliceosomal complex formation. Both activities could be reconstituted by the addition of purified recombinant galectin-1 or galectin-3. In contrast, *immuno*depletion of galectin-1 and/or galectin-3 did not reduce splicing activity or spliceosome formation. However, immunodepleted extracts retained their sensitivity to galectin-specific saccharide addition. Analysis of nuclear proteins bound to lactose-agarose revealed four polypeptides of 42, 36, 29 and 14 kDa. The latter two were identified by Western analysis as galectin-3 and galectin-1, respectively. One or both

of the other proteins many represent uncharacterized nuclear galectins.

These data suggest that the splicing activity of nuclear galectins is functionally redundant. Further, the ability of galectin-1 to restore splicing activity to galectin-depleted extracts suggests that only domains containing the carbohydrate recognition site of galectins are required for splicing function.

S28. 10.50am

Galectin-1 Binds to Integrins on Vascular Smooth Muscle Cells and Inhibits their Migration

D. N. W. Cooper[1,2], V. S. Cannon[1], E. Farrell[1] and R. Clyman[3]
Departments of [1] Psychiatry, [2] Anatomy and [3] Pediatrics, University of California, San Francisco, CA 94143-0984, USA.

Galectin-1 (formerly called L-14, galaptin, etc.) is a soluble, galactoside-binding lectin expressed in multiple mammalian tissues. We have found that galectin-1 is expressed at extremely high levels in vascular smooth muscle cells (SMC) (250 $\mu g\, g^{-1}$ wet weight of sheep neonatal aorta). There, as in many other tissues, this protein appears to be concentrated in basement membranes. Addition of recombinant galectin-1 to SMC in culture had no effect on proliferation and had little effect on substrate adhesion, but almost eliminated migration on laminin, fibronectin or collagen. We postulate that this inhibition results from modulation of integrin receptor function, because integrins, including $\alpha 5\beta 1$, are clearly the predominant glycoconjugate ligands for galectin-1 on the SMC surface. Expression of galectin-1 is greatly decreased in migratory SMC responsible for vascular remodelling during closure of the ductus arteriosus or intimal thickening in response to angioplasty injury. Therefore, one physiological function of galectin-1 may be to modulate integrin activity so as to restrain vascular SMC migration in stable mature tissues.

S28. 11.05am

Tissue Distribution and Structural Analysis of Galectin-8

Y. R. Hadari, K. Rosenblum, R. Lamprecht, M. Eisenstein, Y. Dudai and *Y. Zick*
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We have recently cloned and sequenced a novel rat galectin named Galectin-8 [1] that is structurally related to Galectin-4. Western blotting using specific antibodies revealed that Galectin-8 is expressed in heart, muscle, liver, kidney and brain. Immunohistochemistry and *in situ* hybridization of adult rat brain slices revealed that Galectin-8 is selectively expressed in neurons of the hypocampus (CA1-3 and dentate gyrus), Purkinje cells of the cerebellum, and specific nuclei in the brain stem. The capacity of Galectin-8 to interact with cellular proteins was studied employing the overlay technique. Galectin-8 binding-proteins of 68, 94, 120 and 150 kDa were identified using this procedure. Finally, a model structure of galectin-8, based upon the known structure of Galectin-1 homodimer (34% sequence identity), was built using the Homology (Biosym) program. A few insertions and deletions were necessary and were all confined to loop regions. Galectin-8 consists of a continuous two 10 and 12-stranded anti parallel β-sheets that extend in a two-fold symmetric fashion across the interface of its N- and C-terminal domains. The carbohydrate binding sites are at the far-end of each domain, and no disulfide links are predicted.

Apparently, Galectin-8 shares the overall structural features of galectins although its tissue and cellular distribution are unique. The presence of Galectin-8 in specialized neurons reflects synthesis by the neurons themselves and suggests that Galectin-8 could play some unique roles in the central nervous system. These effects could be mediated through its interactions with specific Galectin-8 binding proteins.

1. Hadari *et al.* (1995) *J Biol Chem* **270**: 3447–53.

S28. 11.20am

Isolation and Molecular Characterization of a Novel Galectin (16 kDa) from the Nematode *Caenorhabditis elegans*

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β-Galactoside-binding lectin (subunit MW, 16 kDa) was purified from the nematode *Caenorhabditis elegans* by an improved worm cultivation, lactose-Ca-TBS extraction and asialofetuin-agarose affinity chromatography. The protein proved not to be a degradation product of the previously reported 32 kDa galectin [1], which consists of two tandemly repeated 14 kDa lectin domains, and thus should be termed a novel galectin in the nematode. The 16 kDa galectin consists of 146 amino acids, of which the N-terminus is blocked, exists as a non-covalent dimer, and the recombinant protein (expressed as a fusion with β-galactosidase α-peptide) showed lactose-sensitive haemagglutinating activity, like typical dimeric mammalian galectins (e.g. human galectin-1). However, it is not thiol-requiring, and has a unique N-terminal sequence; 17-amino acids longer than human galectin-1, though its function is not clear presently. Moreover, distinct from the 32 kDa galectin, the 16 kDa lectin was also extracted with galactose and melibiose, but to a lesser degree than lactose. Based on other biochemical features, the presence of isolectins in the known animal species will be discussed.

1. Hirabayashi *et al.* (1992) *J Biol Chem* **267**: 15485.

S28. 11.35am

Catalytic Lectins ('Leczymes') of Frog Eggs: Mechanism of Tumouricidal Effect

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Sialic acid-binding lectins (SBLs) that inhibit experimental tumour growth were found originally in frog eggs [1, 2]. The amino acid sequences of these lectins from *Rana catesbeiana* (cSBL) and from *R. japonica* (jSBL) show a remarkable homology with pancreatic ribonuclease, and these SBLs in fact display ribonuclease activity [3, 4]. Thus, these SBLs are novel catalytic lectins (termed 'leczymes') with defined carbo-

hydrate-binding specificity as well as defined enzyme substrate specificity. In order to investigate the tumouricidal mechanism of cSBL, we established cSBL-resistant clone RC150 from mouse leukaemia P388 cells. P388 and RC150 cells were equally agglutinated by cSBL. However, growth of only P388 (not RC150) was affected by cSBL. Internalization of dansylcadaverine-labelled cSBL was observed only in P388 (not RC150), suggesting that the internalization mechanism is defective in RC150 cells. Only P388 (not RC150) showed rRNA degradation when incubated with cSBL. Decreased concentration of intracellular Ca^{2+}, decreased protein kinase A activity, and increased protein kinase G activity were observed in P388 but not RC150. We conclude that the tumouricidal effect of leczyme is based on rRNA degradation following receptor binding and internalization, and associated changes in signal transduction.

1. Kawauchi H *et al.* (1975) *Experientia* **31**: 364–5.
2. Nitta K *et al.* (1987) *Cancer Res* **47**: 4877–83.
3. Nitta K *et al.* (1993) *Glycobiology* **3**: 37–45.
4. Nitta K *et al.* (1994) *Cancer Res* **54**: 920–7.

S28. 11.50am

MR60 is an Intracellular Mannose Specific Membrane Lectin which Shuttles Between the Endoplasmic Reticulum and the Golgi Apparatus

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Glycobiologie. CBM CNRS, Rue Charles Sadron, F-450710 Orléans Cedex 02, France.

A mannose specific membrane lectin, with an M_r at 60 000 in polyacrylamide gel electrophoresis under reducing and denaturating (SDS) conditions, has been isolated from human myelomonocytic cells by affinity chromatography [1]. This lectin, designated as MR60, induces a sugar selective aggregation of beads coated with mannosylated serum albumin (ManBSA) but not beads coated with sugar free BSA or glucosylated BSA. MR60 is not present at the cell surface but is localized in cytoplasm organelles as shown by confocal microscopy, using either fluoresceinylated ManBSA or a monoclonal antibody raised against the purified lectin [2]. MR60 was digested with endoproteinase AspN and several peptides were sequenced. Complete sequence was deduced from cDNA [3].

MR60 is not related to any known mammalian lectin but is identical to ERGIC53 [4]; ERGIC53 is a membrane protein shuttling between endoplasmic reticulum and the Golgi apparatus. MR60 could be involved in the traffic of glycoproteins between those compartments. MR60 under non reducing conditions has an M_r of 120 000; in the presence of urea and under reducing agents two protomers were identified in electrofocusing. Therefore, MR60 could be either a homodimer or a heterodimer. The characterization of the second protomer is under way.

1. Pimpaneau *et al.* (1991) *Carbohydr Res* **213**: 95–108.
2. Carpentier *et al.* (1994) *Glycoconjugate J* **11**: 333–38.
3. Arar *et al.* (1995) *J Biol Chem* **270**: 3551–53.
4. Schindler *et al.* (1993) *Eur J Cell Biol* **61**: 1–9.

S28. 12.05pm

Conclusion

S28 POSTERS

S28

A 32 kDa Galectin Composed of Two Tandemly Repeated Homologous Domains in The Nematode *Caenorhabditis elegans*

Y. Arata[1], J. Hirabayashi[1], Y. Akimoto[2], H. Hirano[2] and K. Kasai[1]
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[2] *Department of Anatomy, Kyorin University School of Medicine, Mitaka, Tokyo, 181, Japan.*

We found a 32 kDa galectin and cloned its full-length cDNA from the nematode *Caenorhabditis elegans* (*C. elegans*) [1], which is a very useful experimental animal for the study of the molecular events underlying differentiation and development. This galectin (N32) showed significant sequence homology to vertebrate galectins. It was composed of two tandemly repeated homologous domains, each consisting of about 140 amino acids.

To study the function of this unique polypeptide architecture, we expressed the whole molecule (N32), the N-terminal domain (Nh) and the C-terminal domain (Ch) in *E. coli*. All of the recombinant proteins were bound to asialofetuin-Sepharose in the absence of metal ions. Only N32 showed haemagglutination activity towards trypsinized rabbit erythrocytes. Gel filtration in an HPLC column suggested that all of them exist as monomers. Both Nh and Ch did not seem to form dimers in contrast to vertebrate proto-type galectins. Comparison of the affinity of N32, Nh, and Ch to asialofetuin-Sepharose by frontal affinity chromatography [2] showed that Ch has much weaker affinity than N32. Nh proved to have very low affinity to asialofetuin.

To characterize the properties of galectin in *C. elegans*, we examined the location of the 32 kDa galectin immunohistochemically by use of anti-lectin antiserum. The lectin was found to be localized most abundantly in the adult cuticle, suggesting that expression of galectin is closely related to the cuticular structure of *C. elegans*.

1. Hirabayashi J *et al.* (1992) *J Biol Chem* **267**: 15485.
2. Kasai K *et al.* (1986) *J Chromatogr* **376**: 33.

S28

Structure/Function of Galectin-1 in Chinese Hamster Ovary Cells

M. Cho and R. D. Cummings
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We have studied the structure and function of an S-type lectin designated Galectin-1 (Gal-1) from CHO cells. This lectin has 14 kDa subunits and has been presumed to be a dimer. A recombinant form of Gal-1 was produced in *E. coli* (designated rGal-1) and mutated form was also produced in which Cys at position 2 was changed to a Ser (C2SrGal-1). rGal-1 was unstable in the absence of reducing agent, but glycoprotein ligand, such as basement membrane laminin, stabilized the activity of rGal-1 in the absence of reducing agent. Both rGal-1 and C2SrGal-1 were found to be active monomers at concentrations below 7 μM and to dimerize reversibly at high concentrations. Dimerization was dependent on both time and protein

concentration, but was unaffected by the presence of haptenic sugars or reducing agents. Using a monospecific polyclonal rabbit antibody to Gal-1, biosynthesis of natural Gal-1 in CHO cells was determined. Much of the Gal-1 (45%) is bound to cell surface glycoproteins, but most of it eventually occurs in a soluble form in a medium. In Lec8 CHO cells, which are unable to galactosylate glycoprotein, Gal-1 was not found on the surface and quantitatively accumulated in the medium in an inactive form. We have also constructed fully active monomeric Gal-1, which was made by mutagenizing amino acids involved in the dimerization contact sites.

Supported by NIH Grant CA37626 to R.D.C.

S28

Identification of Galectin-3 Receptors on Macrophage Cell Surface

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Galectin-3 is a galactose-specific carbohydrate binding protein of 30 kDa. Macrophages also express the lectin, known as Mac-2, and the surface expression of this lectin has been shown to be developmentally regulated probably through the regulation of terminal α-galactosyl residues of surface glycoproteins that capture the lectin at the cell surface [1, 2]. We have now studied the macrophage cell surface glycoproteins that have affinity for galectin-3. Macrophage cell line WEHI-3 was lysed with CHAPS and the cell extract was passed through a galectin-3 affinity chromatography column and the binding fraction specifically eluted from the column with lactose. In order to identify the glycoproteins responsible for binding lectin on the macrophage cell surface, intact WEHI-3 cells were biotinylated with a cleavable biotin reagent (HNS-SS-biotin) before preparation of the cell extracts and lectin affinity chromatography; the cell surface expressed lectin-binding glycoproteins were isolated by affinity chromatography on streptavidin agarose. SDS-PAGE of specifically eluted fractions from affinity chromatography showed major components of 172, 125, 100 and 88 KDa. N-terminal amino acid sequencing and additional internal sequencing obtained by chemical cleavage of purified glycoproteins followed by SDS-PAGE separation of the products showed that lysosome associated glycoprotein-1 (LAMP-1), Mac-1 α-subunit (CD11b) and Mac-3 are included in these glycoprotein components. Further characterization is in progress.

1. Sato S, Hughes RC (1994) *J Biol Chem* **269**: 4424.
2. Sato S, Hughes RC (1994) *Eur J Immunol* **24**: 216.

S28

Expression of the Carbohydrate Recognition Domain of Bovine Conglutinin in *E. coli*

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[2]*Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565, Japan.*

Conglutinin is a bovine serum protein and was firstly described as a vertebrate lectin. These lectins are likely to play a important role in basal immune defence against microbial pathogens. We have cloned and characterized the cDNA encoding a bovine conglutinin (BKg). In this study, we tried to express a recombinant BKg in an *E. coli* system and characterize its biological activities compared with native lectins. A 497 bp DNA fragment including the neck region and the carbohydrate recognition domain (CRD) of BKg was amplified by RT-PCR. The cDNA was transferred to the bacterial expression vector system (pRSET-A) and stable transfectants with high levels of the conglutinin production were obtained. SDS-PAGE and Western blotting analysis showed a recombinant protein of 27 kDa. The recombinant BKg fragment (rBKg-CRD) had the binding activity against mannan coated on a plastic plate, as well as native conglutinin. This lectin can also bind to mannan-Sepharose column and inhibits the haemagglutination of influenza A viruses. These results suggested that the recombinant conglutinin, without more than 90% of the collagenous domain, might become a native form and have similar biological activities.

S28

Galectin-6: A New Mammalian Galectin

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We have discovered another mammalian galectin, galectin-6, which is 85% identical to galectin-4 on the amino acid level, but lacks a stretch of 24 amino acids in the link region. The two galectins are not the result of alternative splicing or allelism, but are encoded by two separate genes. Both are expressed in the intestine and colon, starting about embronic day 14.5 and continuing into adulthood. The upstream region of the isolated gene (*LGALS6*) exhibits several possible regulatory elements that could produce this tissue specificity. In addition, there appear to be two promoters upstream of the initiation codon, which could operate under different physiological conditions. The gene is organized into 8 exons and has a large GT dinucleotide repeat in the 3′ untranslated region, right after the polyadenylation signal. The genes encoding galectins-4 and -6 both map to the same region of mouse chromosome 7, 3.2 centimorgans from the *apoE* gene.

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S28

An NMR Study of the Interaction of *N*-Acetyllactosamine and Lacto-*N*-*neo*-tetraose with Recombinant-Derived Galectin-I (L-14)

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The soluble 14 kDa vertebrate galectin-I, also known as L-14, exhibits a strong binding affinity towards poly-*N*-acetyllactosamine oligosaccharides, which may be relevant in cell adhesion to laminin [1]. X-Ray diffraction studies of galectin-I/carbohydrate complexes have described the structural features of the binding site for saccharides ending in a β1,4 galactosyl residue at the non-reducing terminus [2]. To examine these structures in solution, and to investigate reported strong interactions with longer poly-*N*-acetyllactosamine oligosaccharides, we have undertaken an NMR study of galectin-I complexed with *N*-acetyllactosamine-type di- and tetrasaccharides. The galectin was overexpressed in *E. coli* transfected with the gene for L-14, and purified in the large amounts required for NMR analysis. Data are presented that identify the portions of the ligands interacting with the protein, and compare the solution and bound conformations of the saccharides.

Research supported by the NIH Resource Center Grant for Biomedical Complex Carbohydrates (P41-RR05351).

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S28

Forssman Disaccharide GalNAcα1-3GalNAcβ is the Specific Ligand of a Galectin 3-Related L-14 Lectin from the Sponge *Geodia cydonium*, but does not Mediate its Binding to Ribonucleoproteins

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The S-type L-14 lectin from *Geodia cydonium* (GCA), which is involved in regulatory mechanisms of cell sorting and adhesion during reaggregation of allogeneic sponge cells, had previously been shown to bind lactose and other β-galactosides. In this contribution the binding specificity of GCA was established to be GalNAcα1-3GalNAcβ as structural component of Forssman glycolipid. Crossreactivities were revealed in the order GalNAcα1 - 3GalNAcβ > GalNAcα1 - 3(Fucα1 - 2)Galβ ≫ Galβ1-3GlcNAc > Galβ1-4Glc. Lectin binding to the Forssman antigen or to blood group A-trisaccharide exceeded that to lactose by at least three orders of magnitude. Cytochemical staining of eukaryotic cells on the light and electron microscopic level revealed lectin binding in the cytosol and in the nucleus, which was only inhibitable with the soluble high affinity ligand, but not with lactose. The nuclear binding of GCA could be ascribed to affinity-isolated 55–70 kDa proteins of ribonucleoprotein (RNP) complexes and was shown to be mediated by the peptide conformation of the ligand. Although GCA-RNP interaction was inhibitable with Forssman glycolipid, the carbohydrate binding site of the lectin is not involved due to the lack of competition by Forssman-specific C-type lectins HPA or DBA. It is concluded that the L-14 lectin binds to the 55–70 kDa components of RNP-complexes via similar mechanisms as reported previously for the interaction of CBP 35 and CBP 70. While CBP 35 did not bind to Forssman glycolipid, an endogenous lectin activity was localized in the nucleoli of eukaryotic cells by staining with polyacrylamide-conjugated Forssman-disaccharide.

S28

Enhanced Galectin-3 Expression in *In Vitro* and *In Vivo* Oncogene-Transformed Cells

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Among various endogenous lectins characterized in different types of tumour cells, the M_r 34 000 β-galactoside specific lectin galectin-3 was found expressed at high levels in proliferating tumours and in tumour cells acquiring metastatic capabilities. We have investigated the expression of galectin-3 in human HOS cells and murine NIH3T3 cells after stable transfection with a *ras* oncogene-containing pSV2neo plasmid. An increase in galectin-3 mRNA content was found in *ras*-transfected cells in comparison with cells mock-transfected or transfected with pSV2neo. Laser-scanning confocal microscopy of *ras*-transfected cells expressing a high level of galectin-3 mRNA stained with anti-galectin-3 antibodies showed a significant increase in the galectin-3 protein. Amongst *ras*-transfected NIH3T3 cells, the expression of galectin-3 reached the highest level in those cells that have lost their growth anchorage-dependence. The expression of galectin-3 was found to be drastically increased in hepatocarcinomas developing in transgenic mice harbouring the large T SV40 antigen under the control of the antithrombin III promoter. These observations suggest that oncogenes are involved in the regulation of galectin-3 expression. The enhanced expression of this lectin may be important in neoplastic transformation and tumour progression.

S28

Expression, Localization and Regulated Secretion of Galectin-4

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Galectin-4 (36 kDa) is a member of a family of β-galactoside-binding lectins, collectively known as galectins. These proteins can be found in a variety of mammalian cell types. We have examined expression, localization and secretion of galectin-4 in various human epithelial carcinoma cell lines. Our results indicate that galectin-4 and its isoforms are abundant in well differentiated polarized human lung, intestinal and ovarian adenocarcinoma cell lines. In contrast, this protein is virtually absent in less differentiated non-polarizing carcinoma cell lines. Galectin-4 is externalized by the well differentiated polarized carcinoma cell lines constitutively to both apical and basolateral cell surfaces. Immunocytochemistry indicates localization of galectin-4 in the area of adherens junctions. Tyrosine kinases are abundant in this region, and indeed galectin-4 is phosphorylated on tyrosine *in vivo*. We have recently cloned human galectin-4 from intestinal adenocarcinoma T84 cell line and found the sequence to contain a putative *src* tyrosine kinase phosphorylation site. In response to the tyrosine phosphatase

inhibitor vanadate, galectin-4 is released mainly at the basal membrane of the cell which suggests that it is involved in cell-substrate adhesion, perhaps contributing to cell spreading.

S28

α1,3-Galactosylated Ligands For Cell Surface Receptors

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We have used recombinant UDP-Gal:Galβ1 → 4GlcNAc α1,3-galactosyltransferase (α1,3-GT) to synthesize a series of α-galactosylated, lactosamine-type oligosaccharides that differ in their degree of branching. Based on product analysis, it was concluded that recombinant α1,3-GT is able to galactosylate each of the various oligosaccharides to completion, independent of their degree of branching. As a result, all non-reducing termini of these compounds consist of the structure: Galα1 → 3Galβ1 → 4GlcNAc-R. The interaction of these oligosaccharides with mammalian lectins was analysed. In *in vitro* binding assays (Dr R.T. Lee, Johns Hopkins University, Baltimore) it was found that the more highly branched derivatives display a high affinity for the galactose-specific hepatic lectin, although the geometry of the oligosaccharides does not seem to allow optimal binding.

By incubation with human milk α1,3-fucosyltransferase (α1,3-FT), the various α1,3-galactosylated compounds were converted *in vitro* into their α1,3-fucosylated derivatives. Depending on the degree of branching all or part of the terminal trisaccharide structures Galα1 → 3Galβ1 → 4GlcNAc were converted into the tetrasaccharide Galα1 → 3Galβ1 → 4[Fucα1 → 3]GlcNAc (a non-charged analogue of the sialyl-Le^x determinant). The α1,3-galactosylated oligosaccharides and their derivatives that carry one or more Galα1 → 3-Le^x determinants are being tested as potential inhibitors of lectin-mediated cell-cell adhesion (collaboration with Dr W.W. Wright and Dr J.H. Shaper, Johns Hopkins University Medical School, Baltimore).

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S28

Galectins as Household Equipment in the Animal Kingdom

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Until our discovery of a 32 kDa galectin in *C. elegans*, galectins had never been found in invertebrates. The existence of a family member even in the nematode means that the origin of this family can be traced back to the Precambrian period, and their fundamental roles have been preserved. Structural studies revealed that their molecular architecture had already been established before the divergence of the protostomes and deuterostomes. A tandem-repeat type galectin was found for the first time in *C. elegans*, but soon after a mammalian counterpart was reported. Our latest finding of a *C. elegans* 16 kDa galectin showed that the proto-type galectin also exists in the nematode. These facts suggest that both the nematode and mammal are equipped with the same set of glycans in spite of the great phylogenic distance. Although it is not yet easy to conclude they have common roles, lines of biochemical and histochemical evidence showed that they are at least involved in the morphogenesis of the skin in both animal phyla. For example, during the development of chick embryonic skin, expression of two types of galectins (14 K and 16 K) are found to be sophisticatedly regulated in terms of both developmental stages and extra/intracellular location. In the cases of both human and *C. elegans*, importance of galectins in skin formation was also demonstrated. The interaction between galectins and glycoconjugates should be one of the most important basic mechanisms in the biological regulation of multicellular organisms.

S28

Gene Structure and Expression Control of Bovine Conglutinin: A C-Type Mammalian Lectin Containing a Collagen-Like Domain

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Conglutinin is a unique bovine plasma protein which mediates the agglutination of the sensitized erythrocyte-solid phase iC3b (conglutination). The protein is identified as a Ca^{2+}-dependent serum lectin specific for *N*-acetylglucosamine and a member of proteins which have a collagen-like domain (collectin). Conglutinin inhibits human immunodeficiency virus (HIV) and influenza virus infections, suggesting that the lectin is involved in host defence. The cDNA structure and gene organization of conglutinin were characterized [1]. The conglutinin gene spanned over 7.5 kb, and the coding region of conglutinin mRNA consisted of seven exons. The long collagen-like domain (55 Gly-X-Y repeats), which is characteristic of conglutinin, was encoded by five separate exons. The neck region and the carbohydrate-recognition domain were encoded by separate exons. The overall exon-intron organization of conglutinin was very similar to those of other collectins, including pulmonary surfactant apoprotein D and mannan (mannose)-binding proteins. A 5′-flanking region of the conglutinin gene was cloned and examined for its promoter activity using luciferase as a reporter gene to elucidate the biological function of conglutinin with respect to the gene structure. The stepwise deletion analysis of the 5′-region demonstrated the presence of a couple of putative regulatory *cis*-acting elements.

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S28

Regulation of the Galectin-1 Promoter by Sodium Butyrate: Evidence for CBF (NF-Y) and Sp1 Involvement

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Galectin-1 has been found in a variety of mammalian cells and tissues where it has been implicated in cell-cell and cell-extracellular matrix adhesion, growth regulation, transformation, and metastasis. Galectin-1 expression is developmentally regulated but little is known about the regulation of its expression. Among 10 differentiation inducing agents, sodium butyrate (2 mM) was found to be the only agent that could induce both differentiation and expression of galectin-1 in the KM12c human colon carcinoma (HCC) cells at the level of mRNA and protein. Because butyrate is present in the colon at concentrations up to 15 mM, it may play a role in regulating galectin-1 expression in normal cells *in vivo* as well. To understand the mechanism of this effect, we analysed the regulation of the human galectin-1 promoter by butyrate in the KM12c cell line after transfection of a −750 to −39 bp promoter sequence and a series of 5′ deletions inserted into a chloramphenicol acetyl transferase (CAT) reporter plasmid. A proximal region including a CCAAT box and an Sp1 site were found to be sufficient to mediate a >20-fold increase in CAT activity after butyrate treatment of these cells. Deletion of the CCAAT element or site-directed mutagenesis of the Sp1 site resulted in nearly a complete loss of butyrate responsiveness. Gel shift and supershift analyses indicated that this region of the galectin-1 promoter binds the highly conserved heteromeric transcription factor CBF (NF-Y) as well as Sp1 and suggests that these factors may function as components of a butyrate-inducible switch in this promoter.

S28

Flow-Cytofluorimetric Analysis of Young and Senescent Human Erythrocytes Probed with Lectins. Evidence that Sialic Acids Control their Life Span

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Comparing the properties of 'young' and 'aged' erythrocytes (RBC) isolated by ultracentrifugation in a self-forming Percoll gradient, we demonstrate: (i) that the sialic acids of membrane glycoconjugates control the life span of erythrocytes; (ii) that the desialylation of glycans is responsible for the capture of the aged erythrocytes; (iii) that the loss of NeuAc is due, according to D. Aminoff's hypothesis, to a double mechanism: (a) peeling off of membrane vesicles and (b) enzyme desialylation. The capture of senescent RBC is mediated by a β-galectin present in the membrane of macrophages. The evidence supporting these conclusions is based on (i) analyses by flow cytofluorimetry of the binding of fluorescein isothiocyanate labelled lectins and (ii) determination of the rate of transfer of NeuAc by sialyltransferases onto young and old RBC.

S28

Spatial and Temporal Expression of Sea Urchin Egg Lectin During Early Embryogenesis

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Echinoderms contain unique lectins in eggs, spermatozoa and coelomic fluid. We have determined the primary structure of sea urchin egg lectin (SUEL) purified from unfertilized *Anthocidaris crassispina* eggs by thiodigalactoside-column and found that it is nonhomologous to any lectin ever found. We studied the localization of SUEL along with early development of the embryo by immunofluorescent and immunoelectron microscopy using a polyclonal antibody raised against SUEL. SUEL was found to be packed in small granules and distributed randomly in the unfertilized egg. It migrated to the peripheral portion of the egg after fertilization and localized in the cortical cytoplasm during the cleavage stages. Immunoelectron microscopic study indicated that SUEL was deposited in the hyaline layer, the extraembryonic matrix, until the gastrula stage. Formaldehyde-fixed rabbit erythrocytes were incubated with fixed eggs and embryos which were preincubated with SUEL. Erythrocytes adhered to the surface of unfertilized eggs and hatched embryos but not to the fertilization membrane, and the adhesion was cancelled by the co-presence of lactose with the SUEL treatment. These results suggest that SUEL is secreted from embryos at a certain stage (possibly between the blastula and gastrula stage) of early embryogenesis and specifically adsorbed to the hyaline layer by interacting with endogenous carbohydrate ligand(s). The temporal modification occurring at the extraembryonic investment might play an important role in dynamic developmental morphogenesis.

S28

Anomalies in the Biodistribution Pattern of Radioiodinated N-Linked Oligosaccharides

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Specific biodistribution patterns for intravenously injected radioiodinated oligosaccharides have been used to analyse the specificity of known receptors and implicate the existence of putative, undiscovered, mammalian lectins. The detection of receptors in whole animals necessitates the use of iodinated N-linked oligosaccharides. These have been prepared by derivatizing the reducing-end to form a glycosylamine which is coupled to Boc-tyrosine to prepare tyrosinamide oligosaccharides that are then radioiodinated using the chloramine-T method.

N-linked oligosaccharides containing multiple terminal NeuAc, Gal, GalNAc, GlcNAc, and Man residues as well as Le^x determinants have been studied in mice. For those that target well known receptors, such as the asialoglycoprotein receptor, ligand affinity can be used to predict the degree of ligand targeting *in vivo*, which ranges from 5–85%. However,

some oligosaccharides demonstrate an unusual biodistribution pattern to the salivary gland, stomach, and kidney. We have found that trace amounts of free ^{125}I arising during sample storage can lead to anomalies in the biodistribution pattern resulting in approximately 5% accumulation in the salivary and stomach. Also, modification of the charge character on the tyrosine moiety by removal of the Boc protecting group leads to a significant amplification in kidney targeting in a carbohydrate independent fashion. These results suggest caution should be exerted in interpreting the biodistribution patterns for iodinated N-linked oligosaccharide in order to identify new lectins.

S28

Expression of the Lectin p33/41 (annexin IV) in Human Cancer Cell Lines

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We have recently reported a new lectin p33/41 isolated from bovine kidney extract and identified as annexin IV [1]. To study the expression and function of annexin IV, we cloned a cDNA for human annexin IV from a cDNA library constructed from the human colon cancer cell line HT29, prepared a recombinant annexin IV as a fusion protein with GST in an *E. coli* expression system, and generated monoclonal antibodies specific for human annexin IV.

Northern-blot analysis showed that HT29 contained 2.1 kb and 3.0 kb mRNAs of annexin IV. The recombinant annexin IV had the binding activity of fetuin or heparin in the presence of calcium as did bovine annexin IV. Two monoclonal antibodies (AS11, AS17) react with human annexin IV in Western-blotting and by immunoprecipitation. FACS analysis of intact or fixed-permeabilized cells stained with AS11 and AS17 showed annexin IV was located in the cytoplasm, but not on the cell surface. However, immunoprecipitation and SDS-PAGE analysis of biotin-labelled cell surface proteins revealed biotinylation of annexin IV, suggesting that a part of annexin IV is expressed on the cell surface as a membrane-spanning protein. These results suggest that annexin IV is located not only in the cytoplasm but also in the plasma membrane and that the antigenic epitopes recognized by AS11 and AS17 are located on the cytoplasmic side of the plasma membrane.

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S28

The Endogenous Lectin, Galectin-1, Utilizes The Extracellular Matrix Protein Merosin and a β-Lactosamine-Containing Glycolipid for Axon Guidance of Olfactory Neurons

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Galectin-1 is a divalent, lactosamine-binding lectin expressed in the vertebrate nervous system. We have previously demonstrated that galectin-1 binds and co-localizes with two ligands in the rat olfactory system: a β-lactosamine-containing glycolipid, and a member of the laminin family. The glycolipid, paragloboside, is expressed on the surfaces of olfactory axons originating in the olfactory epithelium (OE) and vomeronasal organ. The laminin family member, merosin, is present in the extracellular matrix (ECM) of axonal pathways leading to synaptic targets in the olfactory bulb. We have shown, in cell culture, that galectin-1 can promote crosslinking adjacent axons and axonal adhesion to the ECM. Immunocytochemical studies reveal that five laminin chains (M and K heavy chains and B1, B2 and S light chains) are expressed in the olfactory system. However M-chain is the only heavy chain associated with axonal pathways. Using reverse transcriptase PCR, we recently cloned and sequenced a 600 bp C-terminal region of rat olfactory M-chain. We also made ^{35}S-labelled RNA probes for *in situ* hybridization analysis of M-chain, B-chains and galectin-1. Both B-chain probes hybridized specifically to the lamina propria and the outer nerve layer of the olfactory bulb. M-chain message is also expressed in the lamina propria and nerve layer, but is particularly abundant within vomeronasal nerve bundles. It is also clear that galectin-1 and M-chain synthesis occurs in different cells, galectin-1 message being concentrated in mesenchymal cells surrounding the olfactory and vomeronasal nerve bundles. These data suggest that this novel adhesion mechanism may play an important role in the formation and maintenance of axon bundles.

S28

Further Studies on the Dual Role of Animal Peptide:*N*-Glycanase as an Enzyme and a Carbohydrate-Binding Protein

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Recently, we reported purification of peptide:N-glycanase (PNGase) to homogeneity from mouse-derived fibroblast cells, L-929, and designated as L-929 PNGase [1]. More recently, we demonstrated that the purified L-929 PNGase has the binding activity to yeast mannan in a carbohydrate-dependent manner [2]. We have now analysed the oligosaccharide-binding activity of L-929 PNGase and the inhibition properties by oligosaccharides of the catalytic activity. At pH 7.0, L-929 PNGase was found to bind strongly with oligosaccharides having triomannosido-N,N'-diacetyl-chitobiosyl structure ($Man_3GlcNAc_2$) (K_d = approx. 10 μM). The minimum structural requirements for inhibition of the L-929 PNGase activity were Man_3 and $GlcNAc_2$. The mode of inhibition of the catalytic activity by Man_3 was characteristic for L-929 PNGase and Man_3 and shown not to inhibit PNGase A from almond and PNGase F from bacteria, while $GlcNAc_2$ was a common inhibitor for these three PNGases studied. The Scatchard analysis and enzyme kinetic studies showed that there exist two binding sites for Man_3-containing oligosaccharides on a native L-929 enzyme which was previously found to be a homodimer of two identical 105 K subunits. The present results, when combined with the previous findings that L-929 PNGase was localized intracellularly as a soluble enzyme and required neutral pH for its

optimal enzymatic activity [2], suggest that L-929 PNGase presumably serves not only as an enzyme involved in non-lysosomal degradation of glycoprotein(s) but also as a carbohydrate-binding protein. The latter may possibly function as a receptor/carrier molecule which can be referred to as intracellular 'lectin'.

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S28

Recombinant Bovine Conglutinin produced by Mammalian Cell

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Bovine conglutinin (BKg), a member of the mammalian C-type lectin family, consists of a collagenous and a globular domain. The globular domain carries the binding activity to a carbohydrate. However, the role of the collagenous domain in BKg is not clear. To elucidate this question, it is essential to construct the expression system of the whole BKg molecule. In this study, we amplified the whole coding region of BKg from bovine liver total RNA by RT-PCR. The amplified cDNA was inserted into the newly constructed mammalian expression vector which was composed of colicin E1 replication origin, bovine papilloma virus genome, neomycine resistant gene and cytomegalovirus promoter for expression of foreign gene. Stable transfectant of mouse fibroblast C127 with G418 resistant phenotype was cloned by two cycles of limiting dilution. The culture supernatants of these clones were examined by Western blotting and found to contain recombinant BKg (rBKg). The MW of the rBKg was determined by Western blotting to be 45 kDa which was 2 kDa larger than native BKg (43 kDa). The haemagglutination activity of influenza A virus was inhibited by rBKg and furthermore, rBKg was demonstrated to be able to bind to mannan-Sepharose column. These results indicate that the rBKg produced in this study has a similar activity to the native BKg. The rBKg will be used for the further elucidation of the native role of the collagenous domain of BKg.

S28

Galectin-1 as Transforming Growth Factor: Intramolecule Disulfide Bridges are Indispensable for Transforming Growth Factor Activity

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We have identified a novel transforming growth factor (TGF) $\gamma 2$ from an avian sarcoma virus-transformed rat cells. Protein sequencing and cDNA transfection studies indicated that TGFγ2 is identical to 14 K galectin-1. Purified TGFγ2 had a potent mitogenic activity for BALB3T3 cells but did not have any sugar-binding activity. When purified TGFγ2 was treated with reducing agents such as 2-mercaptoethanol, however, the mitogenic activity was diminished but the sugar binding activity contrarily appeared. Chemical modification of sulfhydryl group in purified TGFγ2 with [^{14}C]iodoacetoamide indicated the disulfide bonds exist intramolecularly. These results suggested that the expression of TGFγ2 activity required the intramolecular disulfide bonds and that these reciprocal effects of the reduction in the activity of TGFγ2/14 K galectin-1 were through the structural changes induced by intramolecular disulfide bond-breakage.

S28

Structure-Function Studies on the Cortical Granule Lectin

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At fertilization in *Xenopus laevis* eggs, a cortical granule lectin (CGL) is released from the eggs and binds to a ligand on the outer surface of the egg envelope to form the fertilization layer; the layer functions as a block to polyspermy. The CGL has been isolated and is an oligomeric glycometalloprotein with a galactosyl binding specificity. We are investigating the structure and role of the glycan moiety which comprises 16% of the glycoprotein. TFMS or N-glycanase treatment produced a 39 K protein. The CGL was recently cloned and shown to have two N-linked glycosylation sites. N-glycanase, under non-denaturing conditions, released >96% of the CGL glycans and resulted in a total loss of carbohydrate binding activity (ELISA, double diffusion, and affinity chromatography experiments). Fractionation of the glycans into high mannose (10%), neutral complex (12%), and acidic complex (78%) was achieved by gel filtration and ion exchange chromatography. The high mannose fraction was composed of Man8 and Man9 structures (NMR and HPLC). The acidic fraction was composed of mono-, di-, tri-, and tetrasialated tetraantennary structures (HPLC). The kinetics of sialic acid hydrolysis corresponded to the presence of two types of sialic acid. TLC analysis of the acid hydrolysed CGL also indicated the presence of two types of sialic acid (NANA and an unidentified derivative). The sialic acid residues were resistant to hydrolysis by *C. perfringens* neuraminidase and partially resistant to *A. ureafaciens* neuraminidase. The immune response in goats was directed against the glycan moiety of the CGL glycoprotein (95%) and specifically against the sialic acid derivative.

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S29. GANGLIOSIDES IN NEUROBIOLOGY

Chairs: Heinrich Rahmann, Robert Ledeen

S29. 9.50am

Introduction

S29. 9.55am

Different Effects of Gangliosides and Ganglioside Antibodies on Voltage-Activated Ion-channel Currents

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While dramatic neuronotrophic effects of gangliosides under certain circumstances have been well documented, the high concentration and complex composition of these cell surface glycosphingolipids especially in nervous tissue suggest their peculiar involvement in basic bio-electrical properties, which however are not yet understood. The assumption of an essential participation of gangliosides in the dynamics of excitation and/or synaptic transmission among nerve cells is being discussed mainly on the basis of their cation binding through negatively charged sialic acid.

To this background we added different ganglioside antibodies (anti-GM1, -GD1b, -GD1a and -GQ1b) while measuring the membrane currents using the whole cell patch clamp configuration with cultured dorsal root ganglion (DRG) neurons of rat pubs. Furthermore we tested the influence of the exogenously applied ganglioside mixture from bovine brain (GM1 21%, GD1a 40%, GD1b 16%, GT1b 19%) on retina ganglion cells from 2-week-old chicken, using the inside-out patch-clamp technique.

Antibodies against the four different gangliosides neither changed the membrane current nor the voltage activated calcium- or sodium-channel current of DRG neurons. These findings do not imply that there are no changes at all at the cell membrane. However, when adding GMix to chicken retina cells one specific voltage sensitive potassium channel was found which was concentration-dependent blocked by gangliosides. These data in addition with other results concerning different effects of exogenous ganglioside application on LTP-formation in mammalian hippocampus and other brain slices are, up to now, still contradictory. However, they may reflect a not yet understood high specificity of ganglioside interactions with functional membrane-bound proteins being involved in the process of membrane excitation.

S29. 10.20am

Regulation by Gangliosides of Calcium/Calmodulin-Dependent Protein Phosphorylation and Dephosphorylation in Living Cells

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We previously showed that gangliosides bind to Ca^{2+}-activated calmodulin (CaM) and regulate CaM-dependent enzyme activity through direct interaction with specific sites of the enzymes *in vitro* [1–3]. These enzymes, especially protein phosphatase 2B (calcineurin) and Ca^{2+}/CaM-dependent protein kinase II (CaMKII) are thought to play important roles in memory formation. To investigate whether gangliosides regulate these enzyme activities in living cells, we have introduced our newly developed real time protein kinase/phosphatase analysis system. This system visualizes the location of an enzyme activity by measuring a change of fluorescence of a fluoroprobe labelled proper peptide substrate for the enzyme. The fluorescence intensity is changed by phosphorylation and dephosphorylation of the substrate. Exogenously added GM1 gangliosides induced calcineurin activity of NG108-15 cells. Calcineurin was also activated by bradykinin or KCl, which increase intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of this cell. GM1 also slightly increased $[Ca^{2+}]_i$ of this cell. Since calcineurin has high affinity for Ca^{2+}/CaM it is thus readily activated by Ca^{2+}/CaM and gangliosides poorly activated by calcineurin. The GM1 activation of calcineurin is most probably through an increment in $[Ca^{2+}]_i$. On the other hand, observation of CaMKII activity by this system suggested that GM1 activated the enzyme in a cultured hippocampal neuron while GM1 did not affect $[Ca^{2+}]_i$ of the cells. Thus, it is possible that gangliosides activate CaMKII through direct interaction with the enzyme in the same manner as *in vitro* activation.

1, 2. Higashi *et al.* (1992) *JBC* **267**: 9839, 9831.
3. Higashi *et al.* (1993) *GlycoXII*.

S29. 10.45am

Inhibitors of Plasma Membrane Ganglioside Sialidase Affect Growth Control and Differentiation in Human Neuroblastoma Cells

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Gangliosides of the plasma membrane are known to be involved in important functions of neuronal cells, including proliferation and differentiation. Therefore metabolism of these glycolipids requires tight control. Whereas the enzymes of ganglioside biosynthesis have been studied in some detail, little is known about the role of ganglioside specific sialidases in cellular processes. Our recent work demonstrated two different ganglioside sialidases in cultured human neuroblastoma cells, a plasma membrane-bound enzyme, which increased about 15-fold during the logarithmic growth phase, and a lysosomal one, whose specific activity remained unchanged [1].

To learn more about the function of the plasma membrane sialidase, we studied the effects of the sialidase inhibitors 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid, heparin and heparan sulfate on various functions of SK-N-MC cells. Upon

addition to the culture medium, the inhibitors caused a characteristic change in the proliferation kinetics, indicating a role of the plasma membrane sialidase in density-dependent growth control of neuroblastoma cells. Additionally, the inhibitors effectively abolished induction of the differentiation marker acetylcholinesterase and of differentiation-associated cAMP elevation, and specifically decreased the binding of iodinated nerve growth factor to the cells. Binding of other ligands such as insulin, insulin-like growth factor I, vasointestinal peptide or neuropeptide Y, whose receptors are also expressed on SK-N-MC cells, was not affected. When the possible existence of a soluble extracellular sialidase was investigated with very sensitive radiometric assays, no such activity was detected in the conditioned medium.

Our results suggest that the ganglioside sialidase of the plasma membrane participates in the control of proliferation and differentiation in this neuronal cell system and that the activity of the enzyme may itself be modulated by cell surface or extracellular components such as glycosaminoglycans.

1. Kopitz J *et al.* (1994) *Biochem Biophys Res Commun* **199**: 1188–93.

S29. 11.10am

Anti-GM1 Auto-Antibodies and Clinical Course in Guillain-Barré Syndrome

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Cross-reacting antibodies against an infectious agent and nerve tissue have been suggested as a pathogenetic factor in GBS. A purported epitope for such antibodies is ganglioside GM1. In the present study the correlation between serum IgM antibodies against GM1 and the clinical condition was investigated in seventeen GBS patients, of whom nine were followed longitudinally. Pooled data from GBS patients showed significantly higher titre than data from 82 blood-donors, median value 52 PB (200-3 PB) and 18 PB (200-0 PB) ($p < 0.001$), respectively. Also GBS patients had higher titres than controls with *C. jejuni* infection, median value of controls 28 PB (136-3 PB) ($p = 0.001$). The anti-GM1 titre was elevated in 14/17 patients ($p < 0.001$). Patients with short-lasting anti-GM1 elevation had a fast recovery whereas patients with slow recovery had a long-lasting anti-GM1 elevation. A linear relationship was found between time until 50% reduction of the anti-GM1 peak ($t_{50\%}$) and the time to significant clinical recovery ($R = 0.91$, $p < 0.01$). Maximal clinical disability was not correlated with high anti-GM1 titres, nor was the absolute titre value a predictor for a long recovery phase. Our data indicate that monitoring of anti-GM1 can predict clinical recovery in GBS patients.

S29. 11.35am

Function of GM1 Ganglioside in the Neuronal Plasma and Nuclear Membranes

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The high content of gangliosides in the neuronal plasma membrane has focussed attention on their functional roles as modulators of enzymes, receptors, ion channels, and other proteins found at that locus. However, recent studies are indicating the presence of gangliosides in various intracellular membranes, including the nuclear. Neuro-2a neuroblastoma cells in the quiescent (undifferentiated) state were permeabilized with saponin and treated with cholera toxin B subunit linked to horseradish peroxidase to reveal minimal staining of the nuclear membrane; however, when stimulated with reagents such as neuraminidase or ionomycin which induce terminal differentiation, staining of the nuclear membrane became very pronounced. Isolation of nuclei from these two groups of cells, followed by ganglioside purification and analysis revealed GM1 to have increased five-fold in the differentiated cells. Primary neuronal cultures from rat superior cervical ganglia and of cerebellar granule cells also revealed increasing neuronal GM1 during the course of spontaneous neurite outgrowth. Preliminary studies with confocal microscopy and $^{45}Ca^{2+}$ suggest the functional role of GM1 in that locus is modulation of Ca^{2+} flux, the net result being to reduce nuclear Ca^{2+} relative to that in the cytosol. This is analogous to the Ca^{2+} modulatory role of GM1 in the plasma membrane which, as revealed by studies with neuroblastoma lines, can either facilitate or inhibit Ca^{2+} influx.

S29. 12.05pm

Conclusion

S29 POSTERS

S29

Developmentally Regulated Expression of GD3 Synthase (α2,8-sialyltransferase) in Rat Brain

Yumiko Watanabe[1], Kiyomitsu Nara[1], Hiroshi Yakahashi[2], Yoshitaka Nagai[2] and Yutaka Sanai[1]
[1]*Department of Biochemical Cell Research, The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan.*
[2]*Department of Histological Chemistry, Mitsubishi Kasei Lifescience Institute, Tokyo, Japan.*

A cDNA (r2,8ST) encoding a rat GD3 synthase was isolated from a rat E15 cDNA library. The cDNA directed expression of the GD3 ganglioside on the cell surface and GD3 synthase activity in the transfected cells. The predicted r2,8ST protein was 342 amino acid typeII membrane protein containing sialyl motifs and found to be 91% similar to its human homologue. Analysis of acceptor specificity of this protein *in vitro* suggested that the protein catalyses transfer of sialic acid via α2,8-linkage to the gangliosides having α2,3NeuAc-galactose moiety. Further we analysed the expression of r2,8ST mRNA in the rat tissues. In the developing rat brain, the expression of the r2,8ST was transiently increased at E15–18. *In situ* hybridization analysis demonstrated that r2,8ST was strongly expressed in ventricular/subventricular zone of CNS and retina in the rat embryo. In the adult, r2,8ST mRNA was detected in cerebral cortex, hippocampus, thalamus and cerebellum. These results indicate that the expression of GD3 ganglioside in brain is regulated by the expression of the GD3 synthase gene and suggests that GD3 is of critical importance for neural development.

S29

Exposure of Cultured Rat Cerebellar Neurons Endogenous Gangliosides is Related to PKC Activity

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Gangliosides undergo lateral phase separation in model and natural membranes with formation of ganglioside-enriched microdomains. Glycosphingolipid segregation is likely to play a role in the cell physiology.

The aim of this work was to study the possible effect of a signal occurring at the plasma membrane level, namely a ligand-receptor interaction, on ganglioside exposure at the cell surface. The experimental model was constituted by granule cells in culture treated or not treated with cytotoxic doses of glutamate. The ganglioside exposure on the plasma membrane was monitored with an enzyme, *Vibrio cholerae* (VC) sialidase, previously shown to be sensitive to the membrane distribution of ganglioside [1]. When cerebellar granule cells were treated with VC sialidase (control), two gangliosides were mainly affected: GD1a and GT1b, giving origin to GM1 and GD1b, respectively. After addition of glutamate or phorbol ester (TPA), inducing translocation and activation of PKC, GD1a and GT1b susceptibility to VC sialidase was lower than in control cells. On the contrary, when the activation of PKC was prevented, by omitting Ca^{2+} in the medium, the hydrolysis of GD1a and GT1b by VC sialidase was not affected.

These data suggest that PKC translocation to the cytosolic side of the plasma membrane is parallelled by a redistribution of gangliosides at the exoplasmic membrane surface, possibly increasing their tendency to form microdomains.

1. Masserini M *et al.* (1988) *Biochemistry* **27**: 7973–78.

S30. ROLE OF GLYCOCONJUGATES IN BACTERIAL AND VIRAL INFECTIONS

Chairs: Elaine Tuomanen, Carl Hellerqvist

S30. 9.50am

Mechanisms of Bacterial Trafficking via the Selectin, Chemokine, and Integrin Systems of Leukocyte Migration

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Bacterial trafficking during infectious diseases is based on cell recognition systems shared with eukaryotic cell trafficking systems. Thus, bacteria can be used as probes to identify new migration mechanisms and, conversely, the carbohydrates that serve as recognition sites can be used to interrupt the progression of bacterial infections. These principles have been applied to the respiratory tract pathogens, *Bordetella pertussis* and *Streptococcus pneumoniae*.

B. pertussis presents two adhesins, one which functions like a selectin and the other with the ability to bind to several leukocyte integrins. The active domains of these proteins inhibit leukocyte trafficking in models of pneumonia and meningitis. Antibody to the integrin binding domain has been used to clone a novel endothelial cell protein involved in leukocyte migration to brain.

Adherence of *S. pneumoniae* is sensitive to the activation state of the target epithelial and endothelial cells, resulting in waves of adherence depending on the presence or absence of cytokines or thrombin. In addition to glycoconjugate-based recognition of resting cells, pneumococci engage a receptor upregulated on activated cells which promotes invasion. This new receptor is the platelet activating factor receptor, a member of the chemokine receptor family. Inhibition of colonization of the respiratory tract by pneumococci can be achieved only by presentation of glyconjugates found on activated cells.

S30. 10.20am

The Globoseries of Glycolipids as Receptors for *Escherichia coli* P fimbriae. Role in Cytokine Activation

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The globoseries of glycolipids are receptors for P fimbriae expressed *Escherichia coli* bacteria. Fimbriae-associated lectins recognize epitopes defined by the Galα1-4Galβ-containing oligosaccharides. Through this specific interaction the bacteria attach to epithelial cells in the large intestine and the urinary tract, and cause urinary tract infection. We have recently demonstrated that fimbrial-glycolipid interactions activate the receptor-bearing cell to produce cytokines. Cytokine mRNA (Interleukin-1, Interleukin-6, Interleukin-8) is upregulated and cytokines are secreted by the cells. The release of cytokines by isolated epithelial cells parallels that seen *in vivo* after experimental urinary tract infection, and in patients with urinary tract infection. Several observations suggest that fimbrial-glycolipid interactions trigger the cytokine response: (1) Isolated P fimbriae trigger cytokine production. (2) Down-regulation of glycosphingolipid expression by PDMP treatment inhibits the cytokine response. We are presently exploring the transmembrane signalling pathways activated by the P fimbriae, which explain the cytokine response.

S30. 10.40am

Structural Definition of Lipoarabinomannan: Its Biological Significance in Host-Parasite Interaction

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All mycobacterial species are endowed with two dominant highly complex polysaccharides, the mycolylarabinogalactan-peptidoglycan (mAGP) complex and the lipoarabinomannan (LAM). LAM in particular, has been implicated to mediate a wide spectrum of immunoregulatory functions and survival of intracellular pathogens such as *M. tuberculosis* and *M. leprae*, the causative agents of tuberculosis and leprosy, respectively. Cumulative studies from this laboratory have led to the recognition of a tri-partite structural model for LAM in which

an immunodominant arabinan is thought to extend covalently in an as yet unidentified manner from a branched mannan core which is in turn directly attached to a phosphatidylinositol 'membrane anchor'. The branched arabinan motifs, variably capped with mannoses, constitute the non-reducing termini of LAM which are assumed to be directly involved in the host-pathogen interaction.

In our continuing effort to completely define the precise structural arrangements of various constituent motifs within the structural model of LAM, as well as strain/species heterogeneity with respect to virulence, novel phosphorylated and acylated moieties have been uncovered and localised. Noteworthy is our concerted approach of various enzymatic degradation, chemical derivatization, separation by HPAE chromatography and FAB-MS analyses which enabled us to map and probe the biological functions of the various structural motifs of LAMs isolated from *M. leprae*, and both nonvirulent and virulent strains of *M. tuberculosis*. With the presence of LAM-reactive CD1-restricted T-cells in leprosy lesions, our recent data indicate that mycobacteria may evade the classical antigen presentation pathways and avoid detection by the host. The relationship of structural features of LAM to these biological properties will be presented.

S30. 11.00am

Possible Role of Membrane Glycolipids and CD4-Free Glycopeptides in Human Immunodeficiency Virus (HIV) Infection of Monocyte-Macrophages

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[2]*CERVI, and* [3]*NSERM U 134, Hôpital Pitié Salpétrière, 75013 Paris, France.*

We have previously demonstrated that human immunodeficiency virus (HIV) envelope glycoproteins (gp120/160) have carbohydrate binding properties for Man/GlcNAc residues [1–3]. We have now investigated the possible role of membrane glycolipids and CD4- free glycopeptides as accessory molecules for HIV infection of macrophages. Using specific mAb, flow cytometry analysis and thin layer chromatography, we show the presence of SGalCer and GalCer at the membrane of human monocyte-derived macrophages (MDM). Anti SGalCer mAb entailed limited (30–40%) but significant inhibition of gp120/160 binding to MDM, but anti GalCer mAb had no effect. In addition, these mAb entailed, at best, limited inhibitory effects on HIV infection of these cells, in contrast to the CD4 mAb Leu3a used as control. These data argue against the possibility that these glycolipids may act as HIV coreceptor on $CD4^+$ MDM. Alternatively, CD4-free glycopeptides prepared from $CD4^+$ monocytic U937 cells, and partially purified by ConA-agarose affinity chromatography, bound gp120 in a mannosyl specific manner and blocked HIV infection of both U937 and CD4 lymphoid CEM cells when preincubated with the virus. Pre-treatment of glycopeptides with N-glycanase abolished their binding to gp120. Thus, surface carbohydrates may play a role in gp120/160 binding to HIV putative coreceptor(s) on $CD4^+$ cells.

1. Seddiki N, Mbemba E, Saffar L, Gattagno L (1991) *Carbohyd Res* **213**: 79.
2. Seddiki N, Benjouad A, Gluckman JC (1992) *Glycobiology* **2**: 429.
3. Ben Younes-Chennoufi A, Baumann N (1993) *Glycoconj J* **10**: 287.

S30. 11.20am

Group B Streptococcus Type II and Type III Neoglycoconjugate Vaccines

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Infections due to group B streptococcus (GBS) are the most common cause of bacterial neonatal sepsis and meningitidis in the United States. Antibody to the capsular polysaccharide protects against invasive neonatal disease, but immunization with capsular polysaccharides fails to elicit protective antibody in many recipients. Recent efforts have focused on increasing the immunogenicity of group B streptococcal polysaccharides, particularly type III, by way of conjugation to tetanus toxoid. These sialic acid-containing polysaccharides can be coupled to carrier proteins by reductive amination either in native form through aldehyde groups introduced into their terminal sialic acid residues or as polysaccharide fragments. Depolymerization of GBS polysaccharides is a difficult task because of their acid-labile antigenically critical sialic acids. Enzymatic digestion with endo-β-galactosidase can be used effectively to break down type III polysaccharide into oligosaccharides. We have developed and report here a new and alternative method of depolymerizing type II and type III polysaccharides by mild deaminative cleavage into antigenic fragments containing terminal aldehyde groups generated from resulting 2,5-anhydro-D-mannose residues. These type II and III polysaccharide fragments have been conjugated to tetanus-toxoid by direct reductive amination, resulting in single-ended neoglycoconjugate vaccines. These conjugates stimulated the production in animals of high-titred type II and type III-specific antibody unlike their respective uncoupled native polysaccharides, in addition the antibodies induced opsonophagocytic killing of type II and type III strains of group B streptococci.

S30. 11.40am

Progress in Developing CM101, a Bacterial Polysaccharide, as an Anti-Neovascularization Drug in Cancer Patients

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CM101, formerly GBS Toxin, a 300 000 Da polysaccharide, is postulated to be associated with ability to induce inflammation in embryonic and pathologic neovasculature. In mice, CM101 binds to tumour endothelium and induces inflammation leading to neovascularitis, thrombosis and infiltration of the tumour by inflammatory cells. This process is initiated within minutes after infusion of 5 pmol of CM101. Complement C3 binds CM101 and data suggest that cytokine release, initiated by CM101 binding to the tumour endothelium, recruits inflammatory cells

which encounter opsonized tumour endothelium. This inflammatory reaction leads to tumour response, extended life expectancy and tumour ablation in some mice bearing small tumours. Neovascularization in wound healing was not affected by CM101.

The proposed mechanism of action of CM101 was corrobortaed in humans participating in a Phase I trial. A time and dose-dependent cytokine cascade (TNF, MIP-1α, IL-8, IL-6 and IL-10) was induced by CM101 infusion in doses from 10 to 50 pmol kg^{-1}. CM101 binds human C3 and C5 *in vitro* and evidence of tumour endothelial targeting was provided by a dose dependent increase in systemic sE-selectin.

Three patients showed tumour response and a biopsy taken after infusion of CM101 showed infiltration of inflammatory cells. No delayed side effects were observed.

Supported by Carbo-Med, Inc.

S30 POSTERS

S30

Nonagglutinating Fimbriae of *Proteus mirabilis* Bind to the Glycosphingolipids Asialo-GM$_1$ and Asialo-GM$_2$

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Proteus mirabilis is a common causative agent of human urinary tract infections. The adherence of *P. mirabilis* to uroepithelial cells and kidney epithelium is mediated by fimbriae and is believed to be an essential requirement in the pathogenesis of *P. mirabilis* infections. *P. mirabilis* adherence is a complex process which may involve a number of fimbrial species including mannose-resistant/*Proteus*-like fimbriae, mannose-resistant/*Klebsiella*-like fimbriae, *P. mirabilis* fimbriae and non-agglutinating fimbriae (NAF) that are expressed under different growth conditions. To gain a better understanding of fimbria-mediated adherence of *P. mirabilis*, we have purified one of these fimbrial species, NAF, and examined its biological role in *P. mirabilis* adherence. Purified NAF were biotinylated (Bt-NAF) and employed in solid-phase binding assays. Bt-NAF bound to gangliosides asialo-GM1 and asialo-GM2 on microtitre plates and on thin-layer chromatography plates. Biotinylated bovine serum albumin demonstrated insignificant binding to these receptors. The binding of Bt-NAF to asialo-GM1 was competitively inhibited by non-biotinylated NAF and anti-NAF monoclonal antibodies. We are in the process of utilizing Bt-NAF and biotinylated whole bacteria to examine the binding characteristics of *P. mirabilis* to a number of cell lines. Characterization and purification of asialo-GM1-like receptors utilized by NAF to bind to host cells will enable us to gain a better understanding of the complex adherence process in order to develop carbohydrate-based anti-adhesives for therapeutic applications.

S30

Immunoaffinity Lipopolysaccaride of Virulent *Shigella sonnei* and its Immunobiological Peculiarities

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Immunobiological properties of *Shigella sonnei* immunoaffinity lipopolysaccarides (LPS) have been assayed. LPS was extracted from keratoconjunctivitis-test positive (virulent) and keratoconjunctivitis-test negative (avirulent) *S. sonnei* strains by the O-specific antibody immunosorbent.

It has been established the intraperitoneal immunization of mice with virulent strain LPS (0.1 mg) occasioned O-specific antibody producing the greatest degree of immunization compared with avirulent strain LPS. In the case of administration of LPS together with sheep erythrocytes (5×10^8 cells) the antibody level to erythrocytes was higher than in the case of using avirulent *Shigella* LPS. There was also a significantly larger mass of lymphnodes in the first case than in the second.

In the case of i.p. injection of avirulent *Shigella* strain together with virulent strain LPS the mice responded with long bacteraemia which was of longer duration than in the case of injection of avirulent *Shigella* together with or without avirulent strain LPS. The prolonged bacteraemia was accompanied by inhibition of a delayed type of hypersensitivity (DTH) which was caused by the xenogenic splenocytes. DTH was not suppressed if mice were treated by avirulent bacteria and LPS of avirulent *Shigella* strain.

The experimental data suggest that virulent *Shigellae* LPS differs from LPS of an avirulent strain with immunobiological properties and function in *Shigella* infection. These properties depend on virulence plasmid and connect with molecular conformation.

S30

New Modification of *Salmonella* Lipopolysaccharide and its Role in Infection

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The influence of *Salmonella typhimurium* lipopolysaccharides (LPS) on cellular immune response (DTH) to xenogenic antigen in mice was studied. The LPS were extracted from bacteria with a phenol water mixture and trichloroacetic acid, and they were also removed from a high-virulent and low-virulent *Salmonella* strain by the O-specific immunosorbent. Additionally the cultural filtrates of these strains were used in the investigation.

It was shown that i.p. injection of the immunoaffinity LPS or cultural filtrates caused DTH suppression in mice. Phenol and trichloroacetic LPS did not have an effect at the DTH level. At the same time the immunoaffinity LPS and *Salmonella* culture filtrates lost immunosuppressive activity after treatment with the trichloroacetic acid or phenol-water mixture, and also after treatment with the O-specific immunosorbent. In the last case eluates from immunosorbents contained the immunosuppressive substance that inhibited DTH in mice.

The results of the investigation show that native LPS plays a more important role in *Salmonella* infection than a phenol or trichloroacetic variant of LPS in that in possessing immunosuppressive properties native LPS can make the elimination of *Salmonellae* from a patient's body difficult and support the infectious process.

S30

Characterization of an Extracellular Polysaccharide from *Epidermophyton floccosum* and its Role in Adherence

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An extracellular polysaccharide was isolated from the culture filtrate of 21-day-old anthropophilic fungus, *Epidermophyton floccosum* (MTCC 613). The crude polysaccharide on passing through Sephadex G-75 was separated into three fractions (F-1, F-2 and F-3), of which the second one (F-2) was obtained in major yield. The fractionated polysaccharides were proteoglycan in nature containing 60–80% carbohydrate and 15–23% protein and gave a precipitin band with certain lectins, viz, jacalin, ConA, wheat germ and *Helix pomatia*. The second fraction was further purified by lectin affinity chromatography using ConA-Sepharose affinity matrix. The two fractions, column-unretarded and column-retarded thus obtained, were also proteoglycan in nature containing 80–90% carbohydrate. Both the fractions were proved to be homogeneous by immunoelectrophoresis against ConA and paper chromatography. The glycan part of the unretarded fraction consists of mainly rhamnose, galactose, glucose and a small amount of mannose whereas column-retarded fraction consists of mainly rhamnose, mannose, glucose and a small amount of galactose. The role of these polysaccharide fractions in the adherence of this fungus during infection to form colonies on human tissues is the subject of discussion in this report.

S30

HIV Envelope Glycans: Influence on Envelope Functions and Routing

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The HIV envelope (Env) precursor gp160 is cleaved into outermembrane gp120 (responsible of CD4 binding) and transmembrane gp41 (induction of membrane fusion). N-glycans (CHO) represent 50% of its MW. They are necessary to create, but not to maintain, Env conformation. Glycosylation inhibitors are therefore used as anti-HIV agents [1]. To study how CHO influence Env properties, the CHO cluster of gp41 was mutated. The mutated (MU) and the normal (WT) gp160 were expressed using vaccinia virus (rVV) in CD4+ cells. Despite a sufficient surface expression of cleaved Env and the binding of gp120 to CD4, mutant gp41 did not induce fusion. Therefore, the gp41 CHO cluster is essential for its function [2].

The routing of MU was compared to that of WT. In rVV infected BHK21 cells, the events occurring in the ER were normal for MU and WT (similar kinetics of folding and oligomerization). In contrast to WT, MU displayed slow transport from cis to medial Golgi while transport to the trans Golgi was impaired (shown by glycosidase analysis). Moreover, cleavage of MU into gp120 and gp41 was reduced by 10 fold but this seemed not due to the involvement of the gp41 CHO *per se* in the cleavage reaction: in the baculovirus system, MU was cleaved when recombinant furin was present. Therefore, the reduced cleavage of MU in BHK21 cells may reflect its impaired routing to the compartment where cleavage occurs. The glycan cluster of gp41 is, therefore, essential for the efficient routing of gp160. MU is an example among few others of proteins that are stuck in the Golgi rather than the ER during biosynthesis [3].

1. Fenouillet E *et al.* (1994) *TIBS*, **19**: 65.
2. Fenouillet E *et al.* (1993) *J Virol* **67**: 150.
3. Fenouillet E, Jones IM (1995) *J Gen Virol*, in press.

S30

A Simple Method for Binding of Microorganisms to Glycolipids Transferred on a PVDF Membrane by TLC Blotting

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The first step in the pathogenesis by enterotoxigenic *E. coli* infection is adherence to the mucosal surface of the small intestine. *E. coli* K99 was demonstrated to bind NGNA-containing glycolipids. In the present study, we developed a simple binding assay for the microorganisms to glycolipids transferred on a PVDF membrane by TLC blotting [1]. A PVDF membrane was blocked with 4% casein/PBS and coated with ^{35}S-*E. coli* suspension. The binding was detected with a Bioimazing Analyzer. Radioactivities were located on the bands corresponding to sialylparagloboside (NGNA) and GM3 (NGNA) with hydroxy fatty acid. The present method has some advantages compared to the overlay binding assay on the HPTLC-plate as follows: (1) simple and rapid; (2) specific with low background; (3) easy handling; and (4) structural analysis of glycolipid ligands can be done by the direct MS analysis [2].

1. Taki T *et al.* (1994) *Anal Biochem* **221**: 312–16.
2. Taki T *et al.* (1995) *Anal Biochem* **225**: in press.

S30

Expression and Biological Activity of Human Salivary Apo-Mucin (MUC7)

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Department of Oral Biology, SUNY at Buffalo, Buffalo, NY, USA.

Previously, we have deduced the amino acid sequence of the 357 residue protein core of the lower molecular weight human salivary mucin [1]. In this study, we have expressed the MUC7 gene product in *E. coli*. The apo-mucin was produced in soluble form in the *E. coli* cytoplasm and purified to homogeneity by sequential gel filtration, ion-exchange chromatography and SDS-PAGE/electroelution. The apo-mucin was compared with the native mucin for bacterial binding in an overlay assay. Two oral (*S. gordonii* and *A. viscosus*) and two respiratory (*S. aureus* and *P. aeruginosa*) strains were tested. The results showed that the carbohydrate moiety of the native mucin was necessary for interaction with the oral strains while the respiratory strains only interacted with the apo-mucin. This finding led us to compare salivary gland and respiratory tissues for MUC7 expression. By RT-PCR and Northern analysis, MUC7 was expressed in human submandibular (SM) gland and to a lesser extent in trachea. Further, we have examined expression of all *MUC* genes (*MUC1-MUC7*) in human SM gland and trachea using the RT-PCR method. We found that SM gland expresses MUC1, 4, and 7 while trachea expresses MUC1, 2, 4, 5, and 7.

In conclusion, since MUC7 is also expressed in trachea, this suggests that it may play a contributory role in pulmonary pathogen colonization of the trachea.

Supported by USPHS Grants DE05650, DE07585, and DE08240.

1. Bobek *et al.* (1993) *JBC* **268**: 20563.

S30

Improved Immunogenicity Using Oligosaccharide-Conjugate Vaccines

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Poor antibody responses to polysaccharide vaccines (thymus independent antigens, TI) are observed in the elderly, the immunosuppressed and children less than 2 years of age. Several investigators are attempting to elicit thymus dependent (TD) responses to a variety of bacterial polysaccharides using protein carriers. Integrity of critical immunogenic epitopes and inconsistency of covalent linkage between the carbohydrate and protein are major limitations with these conjugate vaccines. We have developed coupling technology to give good reproducibility with the carbohydrate to protein ratio of conjugates. Our polysaccharide conjugates elicited non-boostable IgM antibody responses, typical of TI antigens. Oligosaccharides hydrolysed from polysaccharides of *Streptococcus pneumoniae* (serotypes 3 and 8) were used to produce prototype conjugate vaccines which elicit immunoprotective IgG antibody responses. The oligosaccharide size (number of repeat units) appears to be critical for this immunogenicity. The carbohydrate epitopes were analysed by inhibition ELISA, HPLC, GC-MS and NMR.

We are currently developing multi-hapten oligosaccharide conjugates to elicit protection to the serotypes of *S. pneumoniae* which cause acute lower respiratory infections, otitis media and bacteraemia in infants. These conjugates and our immunization regime are designed to reduce antigenic competition and the carrier suppression phenomenon observed with multiple high dose administration of protein carriers. Opsonization results demonstrating efficacy of prototype oligosaccharide conjugate vaccines will be presented.

S30

Enzymology of Human Milk: Fucosyltransferases and Fucosidases

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The fucosyloligosaccharides are a significant fraction of human milk that display great structural complexity and individual heterogeneity. As we have been shown, fucosyloligosaccharides inhibit the stable toxin of Escherichia coli *in vivo* and *in vitro* and also inhibit adherence by invasive strains of *Campylobacter jeuni* to their target cells. The biosynthesis and degradation of fucosyloligosaccharides is controlled by fucosyltransferases (FT) and α-L-fucosidases (FS). However, little is known about the distribution and roles of FT and FS of human milk. The purpose of this study was to characterize the properties, specificity and variation in activity of these enzymes among individuals and over the course of lactation. Multiple forms of FT were found in human milk, by isoelectric focusing, that differ in their acceptor specificity to oligosaccharide acceptors: lactose and *N*-acetyllactosamine. At the concentration of lactose approximating that found in human milk, the major product of the FT reaction was 3-fucosyllactose which was identified by HPLC and by TLC autoradiography. FT was found in both soluble- and membrane-bound forms. In contrast, only one molecular form of FS was found in soluble fraction of milk and was more pH- and thermostable than FT. High individual variability exists for both enzyme activities, but more for FT (472 ± 64 pmol per mg protein per h) than for FS (83 ± 3 nmol per mg protein per h). FS activity increased in milk over the course of lactation, whereas FT activity decreased and then stabilized. The relationship between these enzymes in milk, intracellular events during lactation, and possibility of extracellular modification of fucosyloligosaccharides will be discussed.

Supported by HD13021.

S30

Regulation of Gb_3 (Shiga Toxin Receptor) Expression on Human Vascular Cells in *E. coli* 0157:H7-Associated Disease

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Shiga-like toxins (SLTs) produced by *Escherichia coli* 0157:H7 bind specifically to globotriaosyl ceramide (Gb_3). This receptor is expressed on vascular endothelial cells (EC), the putative target of the SLTs in renal disease of humans. Expression of Gb_3 by human umbilical ECs was induced by bacterial lipopolysaccharide (LPS) or the host cytokines TNF and IL-1, making the ECs more sensitive to SLT. Signal transduction pathways involved in the Gb_3 induction process were not affected by modulators of cyclic AMP, intracellular calcium, or eicosanoid metabolism. Protein kinase C (PKC) activators/inhibitors enhanced Gb_3 induction. The PKC inhibitor calphostin C was capable of directly inducing Gb_3 and sensitizing ECs to SLT. NF/kB, a transcriptional activator downstream of PKC became activated when ECs were incubated with LPS, TNF, or IL-1. Human renal ECs that produce Gb_3 constitutively also contained activated NF/kB. These data suggest PKC and NF/kB involvement in the induction of Gb_3 pathway glycosyltransferase enzymes.

S30

Isolation and Identification of Glycosphingolipids from Mouse Urinary Tract, Recognised by P-fimbriated *E. coli*

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Mouse is being used as a model of human urinary tract infection. P-fimbriated *E. coli* recognize and bind to specific receptors on the cell surface. Differences in binding pattern between mouse and man may be of relevance and we have therefore investigated receptor-active glycoconjugates in these species.

Glycosphingolipids have been isolated by us from mouse kidneys, urethers, and bladders (including urethrae). Binding of a clinical isolate and recombinant strains (expressing class I, II and III adhesins) of uropathogenic P-fimbriated *E. coli* to these glycolipids were studied. A series of glycolipids with Galα4Gal in common, as earlier shown to be recognised by these bacteria, were identified by use of specific monoclonal antibodies, fast-atom bombardment and electron-impact mass spectrometry, and proton nuclear magnetic resonance spectroscopy. The mouse kidneys contained galabiosylceramide, globotriaosylceramide, globoside, the Forssman glycolipid, Galβ4-GlcNAcβ6(Galβ3)GalNAcβ3Galα4Galβ4GlcβCer, and Galβ4-(Fucα3)GlcNAcβ6(Galβ3)GalNAcβ3Galα4Galβ4GlcβCer.

The mouse urinary tract organs contained several glycolipids that were receptor-active for P-fimbriated *E. coli*. However, the binding pattern for mouse kidney glycolipids differed from that for kidney glycolipids of man and monkey. In particular, the dominant eight-sugar glycolipid in the mouse was not detected in the primates.

S30

Human Serum Contains a Chitinase

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Since 1988 an endoglucosaminidase – provisionally named MU-TACT hydrolase – is known that hydrolyses the artificial substrate 4-methylumbelliferyl-tetra-*N*-acetylchitotetraoside (MU-$[GlcNAc]_4$) [1–3]. The biological function of the enzyme was unknown. Evidence is presented here, showing that this endoglucosaminidase from human serum is in fact a chitinase. The enzyme is clearly different from lysozyme. The facts sustaining this finding are: (i) the identification of the products formed from MU-$[GlcNAC]_3$ as $[GlcNAc]_2$ and $[GlcNAc]_3$; (ii) the chitinase substrates chitin and ethylene glycolchitin can be degraded by the enzyme; (iii) the chitinase inhibitor allosamidin also inhibits the action of MU-TACT hydrolase from human serum; (iv) the enzyme did not hydrolyse the lysozyme substrate *Micrococcus lysodeikticus*.

The enzyme also occurs in rat liver. It was demonstrated that upon Percoll density gradient centrifugation the rat liver enzyme distributed parallel to the lysosomal marker enzymes β-*N*-acetylhexosaminidase and β-galactoside, indicating a lysosomal localization for this enzyme.

It is proposed that the enzyme functions in the hydrolysis of chitin, to which mammals are frequently exposed during infection by pathogens.

1. Den Tandt *et al.* (1988) *Int J Biochem* **20**: 713–19.
2. Den Tandt *et al.* (1993) *Int J Biochem* **25**: 113–19.
3. Overdijk *et al.* (1994) *Int J Biochem* **26**: 1369–75.

S30

Mucin-binding Adhesins from Non-Piliated *Pseudomonas aeruginosa*

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Airway colonization by *Pseudomonas aeruginosa* (*P.a.*) is responsible for lung infection in patients with cystic fibrosis (CF). *P.a.* binds to human airway mucins and its outer membrane proteins (OMP) are involved in this binding. In CF, the specificity of colonization by *P.a.* might be related: (i) to mucin carbohydrate abnormalities; or/and (ii) to modifications in the expression of adhesins related to alterations in the airway surface fluid.

The present work was designed to compare the expression of mucin-binding adhesins in the OMPs from two non-piliated strains of *P.a.* (PAK-NP, 1244-NP) in culture conditions which may bear similarities to those found in the respiratory tract. Strains were grown: (i) in a minimum medium (M9) (osmolarity = 210 mOsm); (ii) in M9 made hyperosmolar with 111 mM NaCl; and (iii) in Tryptic Soy Broth (TSB) (osmolarity = 300 mOsm). The OMPs were extracted and the mucin-binding adhesins were detected with ^{125}I labelled airway mucins according to [1].

All the mucin-binding adhesins expressed in TSB had a molecular mass less than 50 K. When the strains were grown in M9, the main difference was the expression of adhesins with a higher molecular-mass. Addition of 111 mM NaCl to M9 did not modify the expression of adhesins in strain 1244-NP, but reduced the number of adhesins in strain PAK-NP.

In conclusion, *P.a.* strains express different mucin-binding adhesins which recognize carbohydrate chains. Their expression varies according to growth conditions (osmolarity and nutrient concentration).

1. Carnoy *et al.* (1994) *Inf Immun* **62**: 1896–1900.

S30

Role of Sulfatides in the Adhesion of *Helicobacter pylori* (*H. pylori*)

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Previously, we have shown that *H. pylori* specifically bound to sulfatides on TLC plates. However, it has not been tested whether sulfatides truly serve as an adhesion receptor for *H. pylori* in the living cells. Therefore, we undertook this study to examine the role of sulfatides in the cellular binding of *H. pylori*, using cultured gastric cancer cells, KATO 3. In order to avoid possible variation between the bacterial strains, we used a type strain of *H. pylori*, NCTC 11637. The bacterial attachment was semi-quantitatively assessed by flow cytometric analysis. Adhesion of *H. pylori* to Kato 3 cells was time dependent and reached plateau within 30 min when bacteria to cell ratio exceeded 100. Bacterial attachment was severely inhibited by pretreatment of Kato 3 cells with anti-sulfatide monoclonal antibody, but not with anti-GM3 nor anti-Le^b monoclonal antibody. Among the glycolipids isolated from Kato 3 cell membranes, *H. pylori* selectively bound to sulfatides. No binding of the bacteria to the membrane proteins transferred to PVD membranes was observed. Immunofluorescent staining of Kato 3 cell with anti-sulfatide monoclonal antibody showed specific fluorescence on the surface of Kato 3 cells. These results suggest the importance of sulfatides in the bacterial attachment of Kato 3 cells. Sulfatides are the most predominant acidic glycosphingolipids in the human gastric mucosa. Furthermore, immunohistochemical study using a monoclonal anti-

sulfatide antibody revealed that they are specifically localized in the gastric surface epithelial cells where *H. pylori* colonizes.

These findings support our hypothesis that sulfatide can serve as an adhesion receptor for *H. pylori* in the human gastric mucosa.

S30

Neisseria meningitidis Lipooligosaccharides Mimic Glycolipids and Glycoproteins in having NeuNAcα2-3Galβ1-4GlcNAc Sequence

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Neisseria meningitidis is a major pathogen causing meningitis in humans. The organism can be serologically divided into 12 types based on lipooligosaccharide (LOS) antigen. *N. meningitidis* LOS may be sialylated or non-sialylated at the nonreducing end. Here, we have characterized the linkage of *N*-acetylneuraminic acid (NeuNAc) in the LOS using lectins. Six of the twelve LOSs (types 2, 3, 4, 5, 7, and 8) bound specifically to *Maackia amurensis* leukoagglutinin which recognizes a terminal NeuNAcα2-3Galβ1-4GlcNAc sequence, but not to *Sambucus nigra* agglutinin which recognizes NeuNAcα2-6Gal sequence. Pretreatment of the LOSs with α2-3 specific neuraminidase from Newcastle Disease virus abolished the reactivity of the LOSs with the lectin. Methylation analysis of the type 2 LOS confirmed that NeuNAc is 2→3 linked to Gal. These results indicate the presence of NeuNAcα2-3Galβ1-4GlcNAc sequence in the six LOSs. This trisaccharide structure in the LOSs mimic human glycolipids such as sialylparagloboside and also glycoproteins such as the Band-3 protein of erythrocytes. The molecular mimicry of the LOS may assist *N. meningitidis* to evade immune defences of the host.

S30

Binding of Salivary Mucins to *Haemophilus parainfluenzae*

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Saliva contains a highly heterogeneous population of mucins, secreted by the different (sero)-mucous glands. The family of salivary mucins comprises high molecular weight mucins ($MW > 1 \times 10^6$ kDa, designated MG-1) and low molecular weight mucins ($MW \sim 125$ kDa, designated MG-2). (Immuno)-chemical analysis has indicated that in saliva subsets of MG-1 are present, having different carbohydrate- and immunochemical compositions. The carbohydrate moiety of MG-1 consists of a wide spectrum of oligosaccharide structures, varying in composition, length, branching and acidity. It is thought that the highly diverse oligosaccharide moiety of MG1 provides a mosaic of potential binding sites for the attachment of bacteria and viruses, allowing their trapping on the mucus blanket and their oral clearance. However, only few studies have demonstrated interaction of microorganisms with MG1, whereas binding of MG2 to several oral microorganisms has often been reported. In the present paper we have focussed on the interaction of MG-1 with oral microorganisms. Using an overlay technique, in combination with immunochemical detection with anti-MG-1 monoclonal antibodies, the oral microflora was screened for the presence of MG-1 binding microorganisms. Using this method it was found that MG1 bound to only a small number of oral microorganisms, one of which was identified as *Haemophilus parainfluenzae*. In contrast, other oral microorganisms, e.g. *Streptococcus, Staphylococcus*, and *Neisseria* spp. were negative for MG-1. MG-1 binding to *Haemophilus parainfluenzae* was abolished after protease treatment of MG-1. In contrast, oxidation of the carbohydrate moiety of MG1 by periodate treatment did not affect the MG1-*Haemophilus* interaction. These results suggest that the colonization of oral mucous tissues by *Haemophilus* spp. is mediated by binding of the bacterium to the protease-vulnerable, unglycosylated peptide moiety of salivary MG-1.

S30

Construction of a Simplified Form of Rabies Virus Glycoprotein

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Rabies virus glycoprotein (RGP) is the only glycoprotein on the viral surface, is the target of viral neutralizing antibodies, and interacts with the host cell receptor. RGP is a 505 amino acid membrane glycoprotein with potential *N*-glycosylation sites at Asn37, Asn247, and Asn319. Due to RGP's role in the biology of rabies, we hope to determine its three-dimensional structure. Thus, we produced a recombinant form of RGP that may be more amenable to crystallization. To avoid using detergents, we constructed a soluble 434 amino acid form of RGP lacking the transmembrane domain (RGPT434). RGPT434 was secreted by transfected cells and was appropriately glycosylated, assembled, antigenic, and immunogenic. Since *N*-glycosylation was required for RGPT434 secretion, we minimized the number of N-glycans of site-directed mutagenesis. Although full-length RGP was expressed at the cell surface if any potential site was glycosylated, RGPT434 was secreted only if Asn319 was glycosylated. Soluble inhibitors were used to simplify the RGPT434 N-glycan structures. Although castanospermine blocked secretion, inhibiting later processing with deoxymannojirimycin or swainsonine had no effect on secretion or assembly. Finally, to simplify purification, 6 histidines were added to the COOH-terminus of RGPT434. This did not affect secretion, antigenicity, or assembly of the resulting glycoprotein. This allowed purification of 1–2 mg of RGPT434 per liter of conditioned medium using Ni + 2-agarose and immunoaffinity chromatography. This soluble form of RGP, with a minimal number of homogeneous N-glycans and an 'epitope' tag, is similar to the extracellular domain of the full-length protein and may be amenable to analysis by X-ray crystallography.

S30

Binding Specificity of *Lactobacillus* to Glycolipids

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An important initial event for intestinal bacteria is to adhere to

cell surfaces in colonizing epithelial tissues. The colonization of useful bacteria on the gut wall is associated with beneficial health effects for the host. *Lactobacillus* is one of the representative useful bacteria in the intestinal tract. So, the binding specificities of two species of *Lactobacillus* were examined with reference to various glycosphingolipids. In thin layer chromatography overlay assays using rabbit antiserum against *Lactobacillus*, two species of *Lactobacillus* were found to bind different glycosphingolipids. *Lactobacillus casei* (IFO 3425) bound to asialo-GM_1 but not GM_1, and also bound to asialo-GM_2 faintly. On the other hand, *Lactobacillus acidophilus* (IFO 13951) bound to globoside but not trihexosylceramide, and also bound to Forssman glycolipid faintly. These findings were confirmed by enzyme treatment of these glycosphingolipids: *L. casei* could bind to the product after sialidase treatment of GM_1 which was not bound to the bacteria, and *L. acidophilus* could not bind to the product after β-*N*-acetylhexosaminidase treatment of globoside. Furthermore, both species were found to bind sulfatide strongly, but no gangliosides.

The bacterial adhesins were also examined. The adhesins of two species seemed to be heat-stable and lectin-like protein existed in the cell surface layer of the bacteria. They were destroyed by protease treatment. However, the two adhesins appeared to be different proteins.

S30

Relevance of Sialylated Structures for the Porcine Paramyxovirus Infectivity

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The porcine paramyxovirus is responsible for neurological and respiratory disease in piglets. The virus recognizes specifically NeuAc lactose molecules and in this work we try to identify the role of the sugar binding specificity for infection. Alteration of glycosylation with tunicamycin and dexynojirimycin showed significant decrease in syncytium formation in cultures with vero cells as opposed to deoxyromanomycin which does not induce alteration of the virus infection. By using lectins with known sugar specificity we also determined that only neurological and bronchiolar tissues which show positive interaction with *Maackia amurensis* interact with the virus. These results confirmed that the porcine paramyxovirus requires sialic acid and sialyl lactose molecules for infection.

This work was financed in part by PAPIIT- and PADEP-UNAM programs.

FRIDAY 25 AUGUST, AFTERNOON

PLENARY LECTURES

S31. 2.40pm

Ganglioside GD2 as a Target for Cancer Therapy

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Murine anti-GD2 mAb 14.G2a and its human/mouse chimeric variant ch14.18 markedly suppressed growth of human tumour xenografts in immunodeficient mice. Both mAbs were effective in clinical phase I trials of neuroblastoma patients resulting in prolonged, partial remissions and several long-term complete remissions with an overall response rate of 30%. To further improve this therapy, recombinant ch14.18-IL2 fusion proteins were constructed and evaluated *in vitro* and *in vivo* in preclinical experiments. The rationale for this approach was to target IL2 to tumour sites expressing ganglioside GD2 to ensure that local T cells would simultaneously recognize their cognate antigens in the context of MHC, while receiving an IL2 activation signal. The ch14.18-IL2 fusion protein significantly enhanced the ability of testing T cells to kill their autologous $GD2^+$ melanoma target cells *in vitro* more effectively than equivalent or higher concentrations of free IL2. The ch14.18-IL2 fusion protein proved more effective than equivalent doses of IL2 in suppressing dissemination and growth of human neuroblastoma in an experimental hepatic metastasis model of SCID mice reconstituted with human lymphokine-activated killer (LAK) cells. The fusion protein was also more proficient than equivalent doses of free IL2 in prolonging the life-span of these animals. These results suggest that recombinant antibody-cytokine fusion proteins may prove useful for future treatment of GD2-expressing human tumours in an adjuvant setting.

S33. 3.10pm

Paroxysmal Nocturnal Hemoglobinuria is Due to a Genetic Defect in GPI Anchor Biosynthesis

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Paroxysmal nocturnal hemoglobinuria (PNH) is a somatically acquired genetic disease caused by defective biosynthesis of the GPI anchor. Blood cells from patients with PNH consist of normal and GPI-anchor-deficient populations. The abnormal erythrocytes lack self-protecting, GPI-anchored complement regulatory proteins, CD59 and DAF, on the surface. This deficiency leads to complement-mediated haemolysis upon intravascular complement activation. Analysis with abnormal lymphocyte lines established from patients with PNH demonstrated that the first step of GPI synthesis, i.e., transfer of GlcNAc to PI, is defective. Somatic cell hybridization with GPI anchor mutants of various complementation groups demonstrated that PNH cells belong to class A among three groups that are defective in the first step. Using a class A mutant cell line, the class A mutant gene, termed *PIG-A* was expression cloned. The *PIG-A* gene is X-linked and encodes a 484 amino acid, ER membrane protein. Within its large cytoplasmic portion there is a conserved sequence that characterizes a glycosyltransferase gene family including a bacterial GlcNAc transferase, RfaK. Taken together with the notion that the first step proceeds in the cytoplasmic face of the ER as indicated from orientation of GlcNAc-PI, PIG-A protein is probably a GlcNAc transferase. Abnormal blood cells from all patients with PNH harbor an inactivating somatic mutation in the

PIG-A gene. The X-chromosomal location accounts for the dominant expression of the mutant phenotype in both male and female cells. Considering that three other GPI biosynthesis genes characterized to date are autosomal, the X-chromosomal location of *PIG-A* would also account for the uniformity of the affected gene.

S31. GLYCOSYLATION AND CANCER

Chairs: Akira Kobata, Jim Dennis

S31. 4.00pm

Alteration in the Sugar Chains of Glycoproteins Produced by Tumour Cells

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Comparative studies of the sugar chains of glycoproteins produced in tumours and their normal counterparts revealed that the malignant alteration in the sugar chains is various. A quite reproducible alteration, however, was found in a particular glycoprotein produced by a particular tumour. Abnormality of *N*-acetylglucosaminyltransferase (GnT) IV in the trophoblasts is the key to altered glycosylation of human chorionic gonadotropin in choriocarcinoma. Enhancement of GnTV plays a major role in malignant alteration of the sugar chains of fibroblast membrane glycoproteins. Furthermore, this phenomenon is found widely in the glycoproteins produced by various tumours with high malignant characteristics such as tumourigenesis and metastasis. Ectopic expression of GnTIII was found in rat but not in human hepatocellular carcinoma, indicating that some of the altered glycosylation in tumours is species specific. Structural alteration in the outer chain moieties of N-linked sugar chains was prominent in the case of normal faecal antigen 2 (NFA-2) produced by large intestine. This glycoprotein contains mono-, bi-, tri- and tetraantennary complex type sugar chains with the type 1 based outer chains as their major outer chains. In contrast, carcinoembryonic antigen, which is the malignant counterpart of NFA-2, contains complex-type sugar chains with type 2 based outer chains. Based on the data obtained by such studies, it is possible to develop novel effective methods for the diagnosis and prognosis of various tumours.

S31. 4.20pm

Reduced Contact-Inhibition and Substratum Adhesion in Epithelial Cells Expressing GlcNAc-Transferase V

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Malignant transformation of fibroblast and epithelial cells is accompanied by increased β1-6 *N*-acetylglucosaminyltransferase V (GlcNAc-TV) activity, a Golgi N-linked oligosaccharide processing enzyme. We have transfected Mv1Lu cells, an immortalized lung epithelial cell line with a GlcNAc-TV expression vector which results in loss of contact-inhibition of cell growth. This effect could be blocked by swainsonine, an inhibitor of Golgi processing enzyme α-mannosidase II. In serum-deprived and high-density monolayer cultures, the GlcNAc-TV transfectants formed foci, maintained microfilaments characteristic of proliferating cells, and also experienced accelerated cell death by apoptosis. Injection of the GlcNAc-TV transfectants into nude mice produced a 50% incidence of benign tumours, and progressively growing tumours in 2/12 mice with a latency of 6 months, while no growth was observed in mice injected with control cells. GlcNAc-TV expressing cells were less adhesive on surfaces coated with fibronectin and collagen type IV, but no changes were observed in levels of cell-surface $\alpha_5\beta_1$ or $\alpha_V\beta_3$ integrins. The larger apparent molecular weights of the LAMP-2 glycoprotein and integrin glycoproteins α_5, α_V and β_1 in the transfected cells indicates that their oligosaccharide chains are substrates for GlcNAc-TV. The results suggest that β1-6GlcNAc-branching of N-linked oligosaccharides contributes directly to relaxed growth controls and reduced substratum adhesion in pre-malignant epithelial cells.

S31. 4.40pm

CMP-NeuAc: Galβ1,4GlcNAc α2,6 Sialyltransferase (β2,6-ST) Gene Transfection Alters the Invasivity of the Human Glioma Cell Line, U-373 MG

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The expression of α2,6 sialyltransferase (EC.2.4.99.1) (α2,6ST) has been shown to be cell-type specific, and appears to modulate a variety of important cellular processes such as CD22-mediated B cell adhesion. It has also been implicated in the progression of human colon carcinoma. We have examined the expression of this sialyltransferase in over 200 human brain tumour specimens and found no malignant gliomas or metastases to the brain which expressed either α2,6ST mRNA or α2,6-linked sialo-glycoconjugates. Glioma cells may therefore provide a model system in which to study the effect of α2,6 containing glycoconjugates on specific biological processes. To study the effect of altered terminal sialylation, we transfected rat α2,6-ST into U-373MG human glioma cells to create stable transfectants which express both α2,6ST mRNA and α2,6-linked sialo-glycoconjugates. The α2,6ST transfected cells grew slower than control U-373MG cells. The transfected U-373MG cells were also much less adhesive to fibronectin-coated plates than controls. Using Biocoat Matrigel Invasion Chambers, a significant (>90%) reduction in invasivity was observed. Gliomas are highly invasive tumours. The data presented here suggest that α2,6ST and sialo-glycoconjugates may regulate adhesive and invasive properties of tumours. Furthermore, these data suggest that changes in the terminal sialylation can have a marked effect on glioma tumourigenicity.

S31. 5.00pm

Cell Surface Exposure of GD3 Ganglioside in Human Melanoma Cells is Inversely Correlated to Both Metastatic Potential and Radiosensitivity

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Large variations in intrinsic radiosensitivity exist among human cell lines and within a given cell line, but the biological basis for such differences is poorly understood. With an experimental model of spontaneous melanoma lung metastases developed in this laboratory, a range of sublines (variants and clones) with different metastatic potential and different ganglioside expression was established from a single poorly metastatic human melanoma cell line M4Be. Variations in the radiosensitivity of seven clones and variants derived from M4Be were also detected, the change essentially arising from differences in the fraction of cells expressing radioresistance. A correlation between intrinsic radiosensitivity of seven sublines and the exposure of gangliosides at the cell surface was found for GD3 by flow cytometry with specific antibodies. Incubation of a GD3-deficient subline with GD3 in medium for 3 days resulted in an increased radioresistance *in vitro*. In contrast, blocking the biosynthesis of GD3 in the parental M4Be cell line with the inhibitor of ceramide biosynthesis Fumonisin B1 reduced its radioresistance *in vitro*. These results suggest that for human melanoma cells, both the metastatic potential and sensitivity to ionizing radiation are related to the biosynthesis of gangliosides.

S31. 5.20pm

Direct Correlation of Cell Surface Sialyl-Le^X, Fucosyltransferase Activity, and Experimental Liver Metastasis of Human Colon Carcinoma Cells

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The levels of human colon carcinoma-associated sialyl-Le^X (sLe^X) antigens inversely correlated with post operative survival of colon carcinoma patients as revealed by retrospective studies. Human colon carcinoma cell lines selected for high levels of cell surface sLe^X (KM12-HX) metastasized to livers when they were injected intrasplenically into nude mice; low expresser cells (KM12-LX) did not. Biosynthetic basis for the increased expression of sLe^X antigens was determined by measuring glycosyltransferase activity. HX cells contained higher α(1,3)-fucosyltransferase (FucT) activity than LX cells. This enzyme(s) transferred fucose to position 3 of *N*-acetylglucosamine in sialylated lacto-*N*-neotetraose but not to position 4 of sialylated lacto-*N*-tetraose. HX and LX cells expressed similar levels of α(2,3)-sialyltransferase specific for lacto-*N*-neotetraose. Increased FucT-III and FucT-VI mRNA was detected in HX cells, although their mutation status was not known. LX cells stably transfected with FucT-III expressed cell surface sLe^X and α(1,3)-FucT activity. These cells were more metastatic to the liver than LX cells when injected intrasplenically into nude mice. They were also strongly adhesive to activated HUVEC. Thus, α(1,3)-FucT should be the key enzyme responsible for the differential metastatic potential and the differential adhesive characteristics of these human colon carcinoma variant cells.

S31. 5.40pm

Hydroxylation of CMP-Neu5Ac is Regulated by Inhibitors in CHO Cells and MDAY-D2 Cells

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CMP-Neu5Ac is converted to CMP-Neu5Ac by the enzyme CMP-Neu5Ac hydroxylase in a multi-enzyme system which includes cytochrome b_5 and an NADH-dependent cytochrome b_5 reducing factor. We have investigated two independent cell lines which have a wild-type (WT) with low levels of Neu5Gc expression and mutants with high levels of Neu5Gc expression. Neu5Gc over-expressing clones of CHO cells were generated by retroviral mutagenesis and selection with WGA, and Neu5Gc over-expressing clones of the MDAY-D2 murine lymphoma cell line were selected by WGA. All thirteen of the CHO-Neu5Gc over-expressing mutants analysed have provirus integration in a genetic locus of approximately 1 kb. Use of an exon trap system has revealed that an exon is present in this 1 kb sequence. When this exon is used to probe a Northern blot with both CHO-WT and Neu5Gc over-expresser RNA, the hybridization intensity in the mutant is approximately half that of WT. This implies that a single copy of the interrupted gene is sufficient to allow increased production of Neu5Gc. We have detected the presence of a low molecular weight cytosolic inhibitor in CHO-WT cytosol which blocks conversion of CMP-Neu5Ac to CMP-Neu5Gc. The inhibitory activity is approximately two-fold higher in CHO-WT than in the CHO-Neu5Gc mutant. A low molecular weight inhibitor has also been detected in the MDAY-D2-WT cells. When CHO-WT cytosol is subjected to size exclusion dialysis followed by reversed phase HPLC chromatography, five distinct cytosolic inhibitors are resolved by a 1.7% per min gradient of H_2O/0.1% trifluoroacetic acid (TFA) to acetonitrile/0.085% TFA. Three of the five species resolved in MDAY-D2 cells are the same as in CHO cells. These data imply that the mechanism for Neu5Gc over-expression is the same in both the CHO and MDAY D2 mutants, and is related to a difference in activity of the inhibitor(s).

S31. 6.00pm

Antibodies and Anti-idiotypic Antibodies in Cancer Diagnosis and Therapy

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A competitive binding immunoassay, employing an epiglycanin-coated plate and an anti-epiglycanin monoclonal antibody, AE3, specific for a glycopeptide sequence containing a Gal(1-3)GalNAc disaccharide, detected advanced human breast carcinoma, at a 90% specificity, with a sensitivity of about 85%. As an aid in cancer management, the assay (the Cod Test) was capable of monitoring the course of breast carcinoma with an

accuracy greater than that of other available tests. The carcinoma-associated antigen, the Human Carcinoma Antigen (HCA), a mucin-type glycoprotein recognized by the AE3 antibody in the Cod Test, appears to be ubiquitous to all types of carcinomas. Four antiidiotypic monoclonal antibodies (AB-2), specific for the hypervariable region of the AE3 antibody, were prepared in the mouse after immunization with AE3 (AB-1). These antibodies could be substituted for epiglycanin in the competitive binding assay. They were capable of inhibiting the binding of AE3 to epiglycanin. Immunization of rabbits with AB-2 produced anti-antiidiotypic antibodies (AB-3). These antibodies, which were able to bind strongly to epiglycanin, were able to inhibit the binding of AB-2 to AB-1. Studies with animals to test the value of the antiidiotypic antibodies as vaccines and therapeutic agents for mammary carcinomas are currently in progress.

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S31 POSTERS

S31

Enzymatic Basis for the High Sialyl-Tn Expression in a Colon Cancer Cell Line

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Sialyl-Tn antigen is not usually exposed on normal colonic mucins but becomes expressed as a cancer-associated antigen. Its presence may indicate a poor prognosis in colorectal and other cancer patients. To understand more about the role of antigen expression in cancer, we have established two clonal cell lines, LSB and LSC, derived from human colonic cancer cells LS174T. These two cell lines have a similar genetic background and have been used for a comparative study of the mechanism underlying sialyl-Tn expression. Mucin from LSB cells was shown to contain oligosaccharides with a wide range of molecular weights. LSC cell mucins, however, contained only Tn and sialyl-Tn antigen. Both cell lines showed activities of a number of glycosyltransferases involved in mucin oligosaccharide biosynthesis but could not synthesize core 3, GlcNAcβ1-3GalNAc-R, found in normal colonic mucins. In addition, LSC cells were shown to lack the ubiquitous enzyme, UDP-Gal: GalNAc-R β3-Gal-transferase. This is the first report of its absence from non-hematopoietic cells. Thus LSB cells are capable of synthesizing and processing core 1, Galβ1-3GalNAc-, and core 2, GlcNAcβ1-6 (Galβ1-3) GalNAc-. However, LSC cells are incapable of forming the common mucin core structures and are therefore committed to short oligosaccharides such as GalNAc and sialyla2-6 GalNAc, explaining the dramatic increase in Tn and sialyl-Tn expression.

This work was supported by the Medical Research Council of Canada and the National Cancer Institute, USA (CA 52491).

S31

Characterization of Certain Lectin Ligands in Sera of Colorectal Cancer Patients

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Changes both in the concentration and structure (increased β1.6 branching of N-linked oligosaccharides, truncation, etc.) may be determined by the use of certain lectins. These assays can be carried out in the pathological tissue samples obtained after surgical operations; however, from a clinical point of view, it is much easier to perform analyses on sera.

We have selected the lectins (commercially available) shown in the following Table, with the aim of ascertaining if they could be useful for detecting changes in NeuAc-, GlcNAc-, Gal- and Fuc-containing glycoconjugates from sera of colorectal cancer patients (obtained 1 day before and 4–7 days after the surgical exeresis) in comparison to control sera. We have optimized the experimental conditions, to avoid nonspecific fixation of the lectins, and for the use of convenient concentrations of both lectins and sera. The results are shown in the Table:

Abbreviated name	*Lectin*		*Results*	
	Source	*Specificity*	*Reactivity*	*Comparison with controls*
SNA	*Sambucus nigra*	Neu5Ac (α2-6)Gal, Neu5Ac (α2-6)GalNAc	Very good	0–25% mean increased value
MAL II	*Maackia amurensis*	Neu5Ac (α2-3)Gal	Good	0–25% mean increased value
WGA	*Triticum vulgare*	GlcNAc $(\beta 1\text{-}4)_3$ > GlcNAc $(\beta 1\text{-}4)_2$ > Neu5Ac	Good	0–12% mean increased value
UEA I	*Ulex europaeus*	α-L-Fuc	Weak	No difference
UEA II	*Ulex europaeus*	L-Fuc (α1-2)Gal(β1-4)Glc	Weak	No difference
SucWGA	*Triticum vulgare*	GlcNAc > Neu5Ac	Very weak	No difference
$GSL\text{-}I\text{-}B_4$	*Bandeiraea simplicifolia*	αGal	Very weak	No difference

In addition, the values obtained have been compared with those of CEA, α-foetoprotein and CA 19.9 of the same patients.

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S31

Mucin Gene Expression, Glycosylation and Sulfation in Ulcerative Colitis

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We have examined the expression of MUC1, 2, 3, 4, 5B, 5C and 6 in colorectal tissue by *in situ* hybridization and mucin glycosylation and sulfation in isolated mucin from ulcerative colitis (UC) patients and controls.

In situ results showed: (1) MUC 2 is the major gene in colonic mucosa; (2) a small increase of MUC 2 appears in UC; (3) MUC 3 expression is normally less than MUC 2, and is normal or slightly increased in UC; (4) MUC 4 expression is elevated in all UC groups; (5) MUC 5B/5C expression was sporadic with some increase of MUC 5C in the UC carcinoma groups; (6) MUC 6 showed intermediate expression in left sided colitis with carcinoma, levels in the other UC groups were lower.

The amino acid composition of the pronase glycopolypeptide from colorectal mucins showed a threonine:serine:proline ratio in good agreement with the MUC 2 ratio predicted from tandem repeat sequence data. There was no significant difference in this ratio between controls and ulcerative colitis groups.

Sulfation was significantly reduced in the glycopolypeptide from the colitis group ($379 \pm 37\ \mathrm{nmol\,mg^{-1}}$ vs $122 \pm 12\ \mathrm{nmol\,g^{-1}}$ in UC, $p < 0.0001$). Overall carbohydrate composition was not different between the groups. The pattern of oligosaccharides released by β-elimination and fractionated on BioGel P6 contained a higher proportion of smaller units, increases in GlcNAc and Neu5Ac in larger oligosaccharides and generally less mannose in UC mucins. These studies indicate that significant modifications to mucin structure during UC relate mainly to oligosaccharide sulfation and appear similar to colorectal cancer mucins.

S31

Oligosaccharide Release From Fresh and Paraffin Wax Embedded Archival Breast Cancers

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To understand cancers we need to relate their biochemistry to natural history, treatment and outcome. Fresh tissue banks are mostly limited to late-stage tumours and autopsies. Early cancers are fixed in formalin and embedded in hot paraffin wax prior to histopathological examination and archival storage. To follow the spectrum of cancer we need to analyse archival tumours whose natural history is known.

Techniques for oligosaccharide release have been established using soluble glycoproteins from fresh tissues. We report here results of oligosaccharide release from paraffin-wax embedded and fresh tumours.

Breast tumours excised at the Middlesex Hospital London in 1991 were used in this study. Following surgery the tumours were divided into two, frozen or fixed in formalin and embedded in paraffin wax. Fifty 5 μm sections were cut from each of the paraffin blocks and dewaxed. Fresh tissues of 0.1 g were taken. N-linked oligosaccharides were released using hydrazine and labelled with 2-aminobenzamide. Sialyated oligosaccharide pools were compared by passing through a GlycoSep C divinylbenzene anion exchange column and neutral oligosaccharides by passing through a BioGel P4 column.

Oligosaccharide profiles obtained from the same fresh and paraffin wax embedded breast tumours show remarkable similarity, both in terms of their sialylated and neutral oligosaccharide profiles.

Our results indicate that whilst the proteins may be denatured, oligosaccharides from glycoproteins in breast tumours are not adversely affected by fixation in formalin and storage in paraffin wax. Using these methods, oligosaccharides from archival clinical specimens may be analysed. Their relation to the patient prognosis and survival can be determined.

S31

A Comparative Study of Peanut Agglutinin and Amarantin Binding to Human Urinary Bladder Tumour Glycoproteins

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The reactivity of two T-antigen specific lectins, Amaranthin (ACA) and Peanut Agglutinin (PNA), was compared by immunohistochemical staining of serial sections of human bladder tumours and by Western Blot analysis of glycoproteins extracted from human bladder tumours prior to and after sialic acid (NeuA) deglycosylation. In addition monosaccharide inhibition tests were performed. In tissue sections of low grade non-invasive bladder tumours the two lectins showed identical staining patterns after neuraminidase treatment. Normal urothelium was stained by ACA prior to NeuA treatment. In Western Blots a mutual band was detected by both lectins. PNA in addition reacted with several other bands after neuraminidase treatment. In monosaccharide inhibition tests ACA- and PNA-binding to different T-antigens (synthetic T-antigen, asialoglycophorin, bladder tumour glycoproteins) was most efficiently inhibited by GalNAc and Gal, respectively. PNA binding was also inhibited by Glc, GlcNAc and GalNAc at high concentrations, while the binding of ACA was only sparsely affected by Glc, GlcNAc and Gal.

S31

Expression and Function of α2,3-Sialyl- and α1,3/1,4-Fucosyltransferases in Colon Adenocarcinoma Cell Lines. Role in Synthesis of E-Selectin Counter-receptors

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Colon carcinoma cell lines COLO 205 and HT-29 adhere to E-selectin via sialyl Lewis x and sialyl Lewis a (sLex and sLea) oligosaccharides linked to cell surface proteins and/or lipids. This adhesion can further be enhanced by TNF-stimulation. These oligosaccharides are synthesized by sequential action of α2,3-sialyl (α2,3-ST) and α1,3/1,4-fucosyltransferases (α1,3/1,4-FT) on existing (poly)*N*-acetyllactosamine chains. mRNAs of two cloned α2,3-ST and four α1,3/1,4-FT are expressed in COLO 205 and HT-29 cells. In functional assays α2,3-ST and α1,3- or α1,4-FT activities were observed in adenocarcinoma cell lysates to exogenous *N*-acetyllactosamine and lacto-*N*-biose acceptors and to their sialylated derivatives, leading to the synthesis of the sialyl-*N*-acetyllactosamine and sLex or the sialyllacto-*N*-biose and sLea, respectively. Furthermore, the inflammatory cytokine TNF was able to enhance some α2,3-ST and α1,3/1,4-FT activities capable of generating E-selectin counter-receptors. Taken together COLO 205 and HT-29 adenocarcinoma cell lines adhere to E-selectin in a TNF-inducible manner via their cell surface sLex and sLea. These cells also express mRNA as well as inducible enzyme activities of several α2,3-ST and α1,3/1,4-FT responsible for the final steps in the synthesis of sLex and sLea.

S31

Novel Anti Tumour Associated Carbohydrate Antigens Monoclonal Antibodies Generated upon Immunization with an Amphibian Mucin

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Five monoclonal antibodies (mAbs) selected on the basis of their anti-carbohydrate specificity were obtained upon immunization of mice with *Pleurodeles waltlii* egg coat mucin, known to express onco-developmental blood group-related determinants (Lewis^y, Lewis^x and A Lewis^y [1]. From immunochemical studies and based on cross-reactivity with other amphibian and mammalian mucins, the five mAbs were shown to recognize distinct complex carbohydrate epitopes. None of the epitopes involved the sialic acid analogue 2-keto-3-deoxy-nonulosonic acid (KDN) present in the newt mucin. A distinctive pattern of binding on breast and colon human tumour cell lines was demonstrated by flow cytometry. One of them, N3 mAb, recognizes a Lewis^y related determinant, which is destroyed upon β-elimination or periodate oxidation. It also reacted with the two colon cancer lines studied (LS 180 and HT 29), and with four estrogen receptor positive breast cancer cell lines (MCF 7, T47 D, ZR 75-1 and BT 20). It did not react with two invasive breast cancer cell lines (MBA 231 and Hs 578T). The four other mAbs, N128, P10, P603 and P631 reacted only with one cancer cell line (HT-29). A specific panel of glycoproteins recognized by the five mAbs was evidenced by Western blot analysis. Xenogenic heavily *O*-glycosylated mucins, such as an amphibian mucin, thus represent an easily available source of immunogenic glycoproteins suitable for the production of sugar-specific human tumour monoclonal antibodies.

1. Strecker *et al.* (1992) *FEBS Lett* **298**: 39–43.

S31

Retrospective Study on the Correlation of *Le*-gene Genotype with CA19-9 Expression and Prognosis of Colon Cancer Patients

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From the random sampling study on more than 200 healthy Japanese individuals, the *Le* alleles were divided into three types, the *Le* allele and the two *le* alleles, *le1* and *le2*, by detection of three missense mutations [1]. The mutant enzymes encoded by *le1* and *le2* alleles are completely inactivated by a single amino acid substitution in the catalytic domain. Individuals who are *le/le* homozygotes have no Fuc-TIII (Lewis enzyme) activity anywhere in their body.

There has been an argument as to whether the Lewis antigens in intestinal tissues, including CA19-9 (sialyl-Le^a; sLe^a) antigen in serum, are products of the *Le* gene or not. It is also of interest to know whether or not the expression level of sLe^a antigen on tumour cells is related to their metastasizing capacity. To solve these questions, genomic DNAs from pathologically-preserved tissues of more than 200 colorectal cancer patients were prepared for *Le* gene genotyping by a PCR-RFLP method recently established by us. Immunohistochemical study showed that the intestinal tissues of all the individuals who are *le/le* homozygotes never stained with antibodies against the type I chains, such as sLe^a, Le^b and Le^a. In conclusion, the Lewis antigens with a type I chain, including the serum CA 19-9 antigen, in the tissues are also the products of the Fuc-TIII enzyme. The prognosis for *le/le* homozygous patients who never express sLe^a antigen is now retrospectively under statistical estimation to compare them with *Le1*-patients in order to clarify the correlation of sLe^a expression with metastasizing capacity.

1. Nishihara S *et al.* (1994) *J Biol Chem* **269**, 29271.

S31

Glycosphingolipid Composition of MCF-7 and MDA-MB-231 Breast Cancer Cell Lines

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Glycosphingolipids of human breast cancer cells were examined to explore the possible linkage between the composition of glycosphingolipids and the progression of breast cancer. The MCF-7 cell line has functional estrogen receptors and is dependent on estrogen for growth *in vitro* and in the nude mouse. The MDA-MB-231 cell line lacks estrogen receptors, is unresponsive to estrogen and anti-estrogens such as tamoxifen

and benzothiophene, and provides a model for more aggressive, hormone-independent breast cancers. MDA-MB-231 and MCF-7 cells contained about the same amount of neutral glycolipids. The major neutral glycolipids in MDA-MB-231 cells were tentatively identified as trihexaosylceramide (CTH) and globoside. MCF-7 cells also contained CTH and globoside, and two other glycolipids as the major neutral glycolipids. The ganglioside content of MDA-MB-231 cells was many times larger than MCF-7 cells. The most abundant ganglioside of MCF-7 cells were GM3, GM2, GM1, and GD1a. MDA-MB-231 cells contained the same gangliosides and also another monosialoganglioside. A striking difference between these two cell lines was the much greater amount of GM3 in MDA-MB-231 cells. This is an important observation in view of the possibility that GM3 may be involved in integrin function in cancer cell progression.

S31

Different Modes of Sialyl-Tn (STn) Expression During Malignant Transformation of Human Colonic Mucosa

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Expression of sialylated carbohydrate antigens such as STn are associated with the cancer phenotype in various human tissues. However, we have observed that in the colon, STn is also present in normal mucosa but the sialic acid moiety is *O*-acetylated making is inaccessible to bind certain mAbs [1]. De-acetylation enabled mAb TKH2 to bind to normal colonic epithelium, but mAb B72.3 did not bind to normal colon tissues under the same conditions. This finding prompted us to reinvestigate the specificities of these two mAbs and the mode of STn expression in colonic tissues.

TKH2 and B72.3 bound to ovine submaxillary mucin in exactly the same manner. Likewise, both mAbs reacted equally well with synthetic clustered STn-KLH. In contrast, only TKH2 bound strongly to monomeric STn-KLH. In addition, the number of epitopes for TKH2 on LS-C cells (STn-positive colon cancer clone) was substantially greater than that for B72.3. Normal colonic tissues were negative with both mAbs but turned positive with TKH2 after de-acetylation. In colon cancers both mAbs were positive to various extents even before de-acetylation.

These results suggest that: (i) mAb TKH2 binds to both single and clustered STn epitopes while mAb B72.3 binds only clustered epitopes; (ii) with malignant transformation, STn expression in colonic epithelial cells undergoes cluster formation and de-acetylation of sialic acid residues.

1. Ogata *et al.* (1995) *Cancer Res* in press.

S31

Sialic Acid Modulates Polymorphism and Bioactivity of Recombinant Human TSH

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Recombinant human TSH (rhTSH) has been recently engineered in Chinese hamster ovary cells to detect metastatic lesions in patients operated on for differentiated thyroid carcinoma. The microheterogeneity, carbohydrate content, bioactivity and immunoreactivity of the product produced in different bioreactors have been compared to those of human TSH from pituitary origin (phTSH).

Carbohydrate composition of rhTSHs was compatible with triantennary N-linked chains terminated with 1.9, 1.1 or 0.5 sialic acid/chain while phTSH mainly contained biantennary chains with sulfated GalNAc and sialic acid. IEF resolved phTSH as five isoforms (pI 6.8–8), rhTSH produced by cells attached to microcarrier beads as six to 10 glycoforms (pI 6.2–8.5) and rhTSH produced in an hollow-fibre reactor as only four species of pI 8.2–8.8. All rhTSH isoforms shifted to pI 8.8 following neuraminidase treatment, indicating that charge heterogeneity of rhTSH is related to sialic acid content. *In vitro* bioactivity of rhTSH proved to be inversely correlated to the degree of sialylation, the asialo product being 10-fold more active than the native hormone. In contrast, *in vivo* bioactivity of rhTSHs augmented with sialylation due to decreased metabolic clearance as a function of their content in sialic acid, showing that this sugar should necessarily be present for the compound to be used as an endocrine drug. Immunoreactivity of rhTSH was investigated using polyclonal and monoclonal antibodies (Abs) raised against the native hormone or synthetic peptides. While rhTSH and phTSH were recognized to a similar extent by anti-protein Abs, they exhibited a different binding pattern to antipeptide Abs. Serial dilution of two anti-α and one anti-β Abs bound rhTSH to a greater extent than phTSH, while two other anti-β Abs displayed similar recognition. rhTSH thus differs from the native protein with respect to several conformational features at the polypeptide surface, which may be responsible for altered intrinsic bioactivity of the product and potential immunogenicity.

It is therefore concluded that, as in the native hormone, rhTSH polymorphism and bioactivity is largely based on sialic acid content. Monitoring the degree of sialylation of the recombinant product is clearly a major challenge in cell engineering for optimizing drug safety and efficacy.

S31

Activities of Four Types of Sialyltransferases and Expression in the Human Colorectal and Rectum Cancer Cells

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Four sialyltransferase activities were compared in cancer (colorectal and rectum) and normal specimens. The ratio of activity of 2,3ST(O) of cancer to normal cells was 5:1.5. On the other hand, the ratio for 2,3ST(N) was 2:1.2. Sialic acid contents of all cancer cells were two to seven times higher than the normal ones. Twenty-two cancer patients were found to be classified into three groups by the contents of mucin type and *N*-glycosidic type glycoproteins. (1) Mucin type sugar moieties increased in cancer cell, on the other hand, *N*-glycosidic moieties did not change. (2) Both *O*- and *N*-glycosidic sugar moieties increased. (3) Mucin type increased but *N*-glycosidic sugar decreased. Sixty four per cent of patients were classified as group 1. The T-antigen type mucin increased both colorectal and rectum cancer. A greater addition of sialic acid to the

non-reducing end of the mucin was observed in colorectal than rectum cancer cells.

S31

Gangliosides of Rat Liver and *N*-nitrosomorfoline Induced Hepatocellular Carcinoma

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Gangliosides were studied in liver and *N*-nitrosomorfoline induced hepatoma subcutaneously growing in an inbred strain of rat LEW-CUB using TLC immuno-detection with monoclonal antibodies. In liver, GM3, GM1a, GD1a and GD1b were the major gangliosides, whereas GM2, GD3, GT1b, GT1aα and GQ1bα were the very minor ones. Composition of hepatoma gangliosides was more complex. A high increase of the expression of GM3, GD3, GD2 and GM1b was observed. Gangliosides of a-pathway GM2, GM1a and GD1a decreased. Only GM1b was present in α-pathway. Fuc-GM1 was not detected. These results suggest that GD3, GD2 and GM1b could be tumour markers for rat hepatocellular carcinoma and raise a possibility of activation of biosynthetic pathway GM3 $\rightarrow$ GD3 ($\rightarrow$ GD2) by nitrosocancerogens. Our findings support the observation of a similar increase in GD3 which has also been observed in diethylnitrosamine induced hepatoma [1].

1. Ye NJ (1990) *Cancer Res.* **50**: 7697–702.

S31

Molecular Identification of a Mucin-Like Protein with Metastasis-Associated Carbohydrate Epitopes in Human Urinary Bladder Carcinomas

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Intense expression of Le^X and related carbohydrate epitopes, namely SSEA-1, *Lotus tetragonolobus* agglutinin (LTA) binding site and sialyl dimeric Le^X (FH6), in transitional cell carcinoma of urinary bladder correlates with the occurrence of lymph node metastasis [1] and poor prognosis of the patient [2]. We prepared monoclonal antibody MM4 using LTA-binding glycoproteins from a BOY urinary bladder carcinoma cell line as the immunogen. MM4 antigen is mostly on a 60 kDa glycoprotein, which carries metastasis-associated carbohydrate epitopes mentioned above, and MM4 itself is a metastasis-associated antigen [3].

To test the possibility that the MM4 antigen is a peptide one, we screened a λgt11 expression cDNA library constructed from bladder carcinoma by using the MM4 monoclonal antibody. Two independent cDNA clones thus obtained encoded different peptide structures, while both had a twice repeated motif of proline-X-proline-X, where X is certain amino acids.

Therefore the tetrapeptide motif is likely to be the antigenic epitope. The deduced protein sequence of the longer cDNA clone was rich in proline, serine and threonine, and was similar to mucin core protein, although an extensive tandem repeat was not present. The deduced protein structure had no clear homology to the known ones. Antibody to the protein reacted with the 60 kDa glycoprotein in the BOY urinary bladder carcinoma cell line. Messenger-RNA of this protein was 2.2 kb in length, and was expressed in bladder carcinomas. These results form the basis of a study on a novel antigenic epitope expressed in metastatic carcinomas, and a mucin-like glycoprotein with metastasis associated carbohydrate epitopes.

1. Shirahama *et al.* (1992) *J Urol* **147**: 1659–64.
2. Shirahama *et al.* (1993) *Cancer* **72**: 1329–34.
3. Matsusako *et al.* (1992) *Histochem J* **24**: 805–10.

S31

Mechanisms Underlying the Aberrant Glycosylation of Polymorphic Epithelial Mucin (PEM) in Carcinomas

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The product of the *MUC1* gene, PEM, is expressed on the apical surface of many simple epithelia. In carcinomas the expression of PEM is upregulated and the glycosylation is different, resulting in the exposure of normally cryptic peptide epitopes and the production of novel carbohydrate epitopes.

To study the mechanisms underlying the aberrant glycosylation of PEM, a cell line MTSVI-7 was developed by SV40 immortalization of normal human milk epithelial cells. This cell line, as shown by differential reactivity of three monoclonal antibodies, glycosylates PEM in a normal manner, compared to breast cancer cell lines T47D, BT20 and MCF7 which show a glycosylation pattern of PEM similar to breast carcinomas *in vivo*.

Using this model system, the mechanisms underlying the aberrant glycosylation of PEM have been studied. Determining the activities of the key enzymes involved in O-linked glycosylation has shown at least a 100-fold decrease in the activity of UDP-GlcNAc:Galβ1-3GalNAc β6-GlcNAc transferase in two breast cancer cell lines. Northern blot analysis has shown that this reduction in enzyme activity is a result of reduced mRNA expression. In addition, an increase in CMP-SA:Galβ1-3GalNAc α3 sialyltransferase activity is seen in all three breast carcinoma cell lines. Increased α2-3 ST mRNA is also observed in BT20 and MCF7.

Although the change in expression and activity of these key glycosyltransferases may, in part, explain the aberrant glycosylation of PEM observed in carcinomas, other mechanisms may be involved. In an attempt to determine if incorrect localization of the enzymes within the Golgi compartments could be involved, the cDNAs of various glycosyltransferases are being tagged with myc or vsvg epitopes and transfected into the cell lines MTSV1-7 and T47D. These transfectants are being analysed by immunoEM to show the positions of these previously unmapped enzymes within the Golgi, and to identify any alterations in the tumour cell lines.

Supported by the Imperial Cancer Research Fund and the Medical Research Council of Canada.

S32. MOLECULAR GENETICS OF HISTO-BLOOD GROUPS AND DISEASES

Chairs: Winifred Watkins, Jerzy Koscielak

S32. 4.00pm

Blood Group MN Precursor Glycoepitopes T and Tn in Successful Preclinical Carcinoma (CA) Detection and Specific Active Breast CA Vaccination

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T (βGal1→3GalNAcα-*O*-Ser/Thr) and its precursor Tn epitope (EP), the immediate precursors of carbohydrates determining blood group M & N specificities, are sensitive, specific panCA markers. CA elicits strong cellular and humoral anti-T autoimmune responses.

Eighty-six subjects suspected of CA were followed: two groups consisting of: (a) 48 patients with seemingly false positive anti-T reactions and negative biopsies/X-rays, 75% of whom developed histological CA within months to >10 yrs (x̄ 6 yrs); (b) 38 breast disease patients with repeatedly negative anti-T reactions, who developed no CA during 2.4–10.7 yrs (x̄ 4.7 yrs). Thus, we detected *pre*clinical CA with anti-T tests and opened a novel avenue for CA treatment and pathogenesis study.

Since 1974 we have vaccinated advanced breast CA patients with: O MN RBC-derived T/Tn Ag adsorbed onto $Ca_3(PO_4)_2$ + typhoid vaccine as hyperantigen. Injection is i.d. at 6–12 wk intervals *ad infinitum*.

Of 20 patients, seven Stage IV, six St III, seven St II, all survived >5 yrs. 12 survived >10 to >18 yrs and four died of CA before 10 yrs; four others alive have not reached 10 yrs, i.e. so far 75% survived >10 yrs. *p*-value of our survival rates, with U.S. NCI Standard Data as control, is for all three stages combined: 5 yr: $<3 \times 10^{-7}$; 10 yr: $<1 \times 10^{-5}$.

An additional 28 breast CA patients are being vaccinated, they are all well but none is as yet 5 yrs post-op.

S32. 4.25pm

Transgenic Mice Expressing a Human α1,3/4Fucosyltransferase in Gastric Surface Mucous Cells – a Potential Model for *Helicobacter pylori* Infection

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[3]*Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St Louis, MO, USA.*

We have characterized an *H. pylori* receptor on the surface mucous cells in human stomach, the carbohydrate blood group antigen Lewis b (Le^b). To examine if Le^b-mediated attachment of bacteria to gastric mucosa leads to infection, a human α1,3/4-fucosyltransferase (α1,3/4FT), encoded by the Lewis blood group locus, was expressed in surface mucous cells of FVB/N mice. Immunohistochemical analyses of the carbohydrates in FVB/N mice showed that the surface mucous cells produced epitopes with a terminal fucose residue, which could act as acceptor molecules in the transglycosylation reaction in which α1,3/4FT generates Le^b-type epitopes. Transcription was regulated by an upstream promoter consisting of the nucleotides −596 to +21 of the non-coding region from the rat liver fatty acid binding protein gene, ensuring tissue-specific gene expression. Transcription of the reporter gene was determined by RTPCR. Expression of the receptor was determined by immunohistochemistry using monoclonal antibodies against Le^b. Clinical isolates of *H. pylori* bound to sections of stomach from transgenic but not from nontransgenic animals. Binding was restricted to cells that expressed Le^b, and could be inhibited in a competitive fashion by soluble receptor analogues, as was the case with human stomach sections. These animals are currently being tested for their ability to be colonized by *H. pylori* under germfree and conventional conditions.

S32. 4.50pm

Expression of the Uropathogenic *Escherichia coli*-Binding Globo-Series Glycosphingolipids Sialosyl Gal-Globoside and Disialosyl Gal-Globoside in Vaginal Epithelial Cells is Dependent on Histo-Blood Group Secretor Status

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[2]*The Biomembrane Institute, Seattle, WA, USA.*

Women with a history of recurrent *Escherichia coli* urinary tract infections (UTIs) are significantly more likely to be nonsecretors of histo-blood group antigens than are women without such a history and vaginal epithelial cells (VEC) from nonsecretor women show enhanced adherence of uropathogenic *E. coli* compared with cells from secretors. Uropathogenic *E. coli* expressing P-related adhesins bind globo-series glycosphingolipids (GSLs) on uroepithelial cells and in the kidney. We extracted GSLs from native VEC collected from nonsecretors and secretors and found that the nonsecretors' VEC selectively expressed two extended globo-series GSLs, sialosyl Gal-globoside (SGG) and disialosyl Gal-globoside (DSGG), which bind cloned and wild-type P-related adhesin-expressing uropathogenic *E. coli*, including $papJ_{96}$, $pap\text{-}2_{J96}$, and pap_{IA2}. Presumably the expression of SGG and DSGG by VEC of nonsecretors is a result of sialylation of the Gal-globoside precursor glycolipid, which we found in secretors' VEC is fucosylated and processed to ABH antigens. In addition, SGG binds representative cloned and wild-type uropathogenic *E. coli* with higher affinity than does globotriaosylceramide. The presence of SGG and DSGG may account for the increased binding of *E. coli* to uroepithelial cells from nonsecretors and for their increased susceptibility to recurrent UTI.

S32. 5.10pm

Point Mutations in the Lewis FUT3 Gene among Caucasians

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The human Lewis gene *FUT3* encodes an α (1,3/1,4) fucosyltransferase responsible for the synthesis of the Lewis a and Lewis b antigens. In order to understand the molecular genetics behind non functional fucosyltransferases we have characterized the *FUT3* gene in 18 Caucasians initially typed as Le(a−b−) individuals. Eighteen Le(a−b+) and 4 Le(a+b−) served as a controls. The *FUT3* gene from three Le(a−b−) individuals was partially sequenced through PCR amplified DNA fragments. Each observed mutation was screened for by PCR and restriction enzyme cleavage or by PCR using sequence specific primers. Five point mutations in the coding sequence i.e. at nucleotides 59, 202, 314, 508 and 1067 have so far been identified. These mutations represent the following amino acid substitutions Leu20 → Arg, Trp68 → Arg, Thr105 → Met, Gly170 → Ser and Ile356 → Lys. Of the 18 Le(a−b−) individuals six typed as $le^{59/1067}le^{202/314}$, four as $le^{202/314}le^{202/314}$ and one as $le^{59/1067}le^{59/1067}$. Additional allelic combinations were $le^{59/508}le^{59/1067}$, $le^{59/202/1067}le^{202/314}$, $le^{59/508}le^{202/314}$ but all Le(a−b−) phenotypes were not explained by their genotypes. Of 18 Le(a−b+) and four Le(a+b−) 16 typed as LeLe, five as $Lele^{202/314}$ and one as $Lele^{59/202/1067}$. A pedigree study of one family of eight Le(a−b+) individuals proved the mutations at nucleotides 202 and 314 to be located on the same allele.

S32. 5.30pm

Molecular Genetic and Biochemical Analysis of the Human Lewis Histo-blood Group System

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The expression of Lewis histo-blood group antigen is determined by the Lewis type α(1,3/1,4) fucosyltransferase encoded in Fuc-TIII gene(*Le* gene) on chromosome 19. The *Le* alleles of the Japanese population from the analysis of more than 200 individuals were divided into three types, *Le* allele, having no mutation, *le1* allele, having the T59G(Leu20 to Arg) and the G508A(Gly170 to Ser) missense mutations, and *le2*, having the T59G and the T1067A(Ile356 to Lys) missense mutations. The frequency of occurrence of *Le*, *le1*, *le2* in the Japanese population was found to be 66, 30 and 4%, respectively. The single amino acid substitution in the catalytic domain, e.g. Gly170 to Ser in the *le1* allele or Ile356 to Lys in the *le2* allele, made the enzyme inactive.

Histochemical and biochemical analyses were performed on the mutant enzymes by using the Fuc-TIII specific monoclonal antibody. Each mutant enzyme was retained in Golgi apparatus in the gene-transfected cells and showed the resistance to protease as much as the active enzyme. Furthermore, the nonfunctional Fuc-TIII enzyme was detected by Western-blotting analysis in saliva samples of *le/le* homozygotes. It was suggested that all mutant enzymes had the folded structure whose mutations might be included in the acceptor or the donor binding site.

The molecular genetic analysis of the secretor gene (*Se* gene), which determines the expression of Le^b and Le^a antigens, is now under progress.

S32. 5.50pm

Loss of ABH Antigen Expression in Human Bladder Cancer, Caused by Downregulated Transcription and Not LOH of ABO Locus

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Loss of tissue ABH antigen expression in bladder tumours is highly associated with an invasive disease course. We have previously demonstrated loss of ABH glycosyltransferase activity in bladder tumours, and found this *in vitro* to be due to loss of translation of the mRNA produced. However, *in vivo*, it could be due to the general (> 60%) loss of heterozygocity of chr. 9q in bladder tumours, as the ABO locus is on 9q34. Based on this we decided to study: (i) LOH of the ABO locus on pure tumour nuclei, separated by FACS according to aneuploidy; and (ii) ABH-mRNA expression by RT-PCR on RNA extracted from clinical specimens and correlated to ABH immunohistochemistry.

LOH of the ABO locus was examined by PCR genotyping of 11 bladder tumours and corresponding leukocytes. Seven tumours were informative and separated by FACS according to DNA content. In two AO cases having two aneuploid subpopulations, the most abnormal showed complete loss of the O allele, leaving the A allele intact.

RT-PCR of ABO mRNA in tissue biopsies showed mRNA to be present in normal control biopsies, and to be correlated to immunostaining in 20 tumour biopsies: ABH antigen positive biopsies had ABO-mRNA, ABH antigen negative biopsies had no ABO-mRNA.

We conclude that loss of ABH antigens in bladder tumours is due to downregulated transcription, and not due to LOH of chr. 9q34.

S32 POSTERS

S32

A Rapid and Simple *ABO* Genotype Screening Method Using a Novel B/O^2 Vs. A/O^1 Discriminating Nucleotide Substitution at the ABO Locus

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An *ABO* genotype screening method discriminating the common alleles A^1, A^2, B, O^1 and O^2 at the *ABO* locus was made possible by the discovery of a novel nucleotide substitution (G1096A) present only in *B* and O^2 alleles. A rapid and reliable single-tube approach using multiplex PCR with four primers amplifying exons VI and VII of the *ABO* genes followed by simultaneous addition of two restriction enzymes was developed and validated in a population of 150 Swedish blood donors.

DNA preparations were all strongly amplified in the multiplex PCR system. The fragments amplified cover exons VI and VII. After simultaneous digestion with *Hpa* II and *Kpn* I a conclusive interpretation of the expected fragmentation pattern obtained could be made in all cases. All genotyping results were in agreement with the serologically determined phenotypes and previously determined genotypes with the exception of one

sample that was genotyped as A^2O^1 but was from an O individual. Further characterization of the allele indicated an A^2 variant. This method is based on the cleavage of exon VII with *Hpa* II and utilizes a novel B/O^2 vs. $A^1/A^2/O^1$ discriminating nucleotide substitution (G1096A). Only A^1 and O^1 cannot be differentiated by this enzyme alone, but this is overcome by a simultaneous cleavage of exon VI using *Kpn* I. This new method not only differentiates *ABO* alleles, including the recently described O^2 allele, but also discriminates A^1 from A^2.

S32

Erythrocyte Epitopes of Blood Group A Antigen are Cleaved at Different Rates by α-*N*-Acetylgalactosaminidase

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Red cell epitopes of blood group A antigen causing haemagglutination with *Dolichos biflorus* (*Db*) lectin are highly sensitive to glycosidic cleavage whereas those causing haemagglutination with anti-A antibodies are more resistant [1]. We have extended these studies to include A antigen epitopes binding complement fixing anti-A antibodies, quantitation of binding by flow cytometric analysis and an α-*N*-acetylgalactosaminidase ('α-GalNAc'ase') purified from culture supernates of the human gut bacterium *Ruminicoccus torques*.

Methods: Aliquots of A_1 and A_2 red cells (RBC's), incubated with α-GalNAc'ase or buffer, were removed at intervals, washed, and tested for loss of binding to anti-A iso- and monoclonal antibodies and lectins (*Db* anti-A_1 and *Ulex europaeus* (*Ue*) anti-H) by haemagglutination titration and flow cytometry. Binding of complement fixing anti-A was measured by an immune haemolysis assay. Neutral glycolipids (GL's) were extracted from HCHO-fixed RBC aliquots, separated by thin layer chromatography, and immunostained for bound anti-A antibody or lectin.

Results: A epitopes binding *Db* were highly susceptible to cleavage by α-GalNAc'ase; simultaneously, *Ue* binding emerged. Cleavage of A epitopes binding complement fixing immune antibody was slightly slower, while those binding haemagglutinating antibody showed slowest and incomplete cleavage, even at 10 h with 10 U ml^{-1} α-GalNAc'ase. Cleavage of A epitopes binding anti-A antibody and *Db* on long chain (>12) GL's occurred within minutes whereas cleavage from short chain (6-12) GL's required 24 h incubation.

Conclusions: Blood group A epitopes on RBC's vary in biological reactivity and rate of enzymatic cleavage, suggesting that subsets mediating different biological functions exist on topographically distinct membrane glycoconjugates.

1. (1991) *Arch Biochem Biophys* **290**: 312–19.

S32

Murine Monoclonal Antibody that Binds to α(1,3/1,4)Fucosyltransferase

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We prepared a murine mAb, FTA1-16, that recognizes α(1,3/1,4)fucosyltransferase (Fuc-TIII). To address the binding specificity of FTA1-16, Namalwa cells were stably transfected with each cDNA of Fuc-TIII, Fuc-TIV, Fuc-TV, Fuc-TVI, or Fuc-TVII, and the transformants were stained by FTA1-16. FTA1-16 specifically stained the Golgi apparatus of Namalwa-Fuc-TIII cells, but not the other transformant cells. This result was confirmed by Western blotting experiment using lysates of the transformants. FTA1-16 could immunoprecipitate a 45 kDa protein, Fuc-TIII, from the Namalwa-Fuc-TIII cells.

To determine the antigenic epitope of FTA1-16, a full length peptide and seven truncated forms of Fuc-TIII were expressed in *E. coli*. FTA1-16 recognized a peptide that contains 22 amino acid residues of the putative catalytic domain of Fuc-TIII. Five amino acid residues in the 22 residues were not shared by the equivalent region of other Fuc-Ts.

These results clearly indicate that FTA1-16 has a strict specificity to Fuc-TIII and will be a practical probe to detect Fuc-TIII. We are now doing an immunohistochemical study to find the tissue distribution of the Fuc-TIII enzyme and its expression in malignant cells. The other Fuc-Ts are considered to share the similar tertiary-structure with Fuc-TIII. The antigenic epitope of FTA1-16 determined in this experiment would be useful for establishing monoclonal antibodies against the other Fuc-Ts.

S32

Detection and Isolation of Plasma Glycoproteins Carrying ABO Blood Group Antigens by Lectins

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Interaction between blood group specific lectins and glycoproteins expressing blood group antigens has not been studied in detail. We have so far found that ABO blood group antigens are covalently bound to von Willebrand factor (vWF), α_2-macroglobulin (α_2M) and coagulation factor VIII among human plasma proteins. We studied the binding of blood group specific lectins to those plasma components and their specificities compared to anti-blood group antibodies. Plasma glycoproteins were transferred to PVDF membranes after SDS-PAGE and subjected to Western blotting analysis using biotinylated lectins and anti-A, -B monoclonal antibodies. Among blood group specific lectins examined (DBA and HPA for type A, BSL-I(B4) and EEA for type B, UEA-I, -II and LTA for type O), HPA (from *Helix pomatia*) was bound specifically to vWF from group A or AB plasma in the absence of GalNAc with almost the same sensitivity as the anti-A antibody used. Anti-H lectins, UEA-I and -II (from *Ulex europaeus*), reacted to vWF from group O plasma better than vWF from group A or B plasma. Other lectins seemed to be nonspecific. Citrated plasma was applied to a HPA-agarose column and the eluate with PBS containing GalNAc was analysed by SDS-PAGE. Several bands reactive to the anti-A antibody including α_2M and vWF were observed in the eluate from group A plasma, whereas those bands were not obtained from group O plasma. The results indicate that HPA, an animal lectin, is useful for detection and separation of plasma glycoproteins carrying blood group A antigen as well as the antibody.

S32

Expression of a Recombinant Human Glycosyltransferase from a Synthetic Gene and its Utilization for Stereospecific Synthesis of the Human Blood Group B Oligosaccharide Antigen

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The human blood group B glycosyltransferase (EC 2.4.1.37), which catalyses the transfer of Gal from UDP-Gal to Fucα-(1-2)Galβ-OR to give the blood group B determinant Galα-(1-3)[Fucα(1-2)]Galβ-OR, has been expressed without its membrane-anchoring domain in *E. coli* using a synthetic gene. The active enzyme was purified from the periplasm using UDP-hexanolamine affinity chromatography. The substrate specificity of the recombinant enzyme was tested using synthetic analogues of the H disaccharide acceptor Fucα(1-2)Galβ-O-$(CH_2)_7CH_3$ and found to be comparable to the enzyme from human sera. Purified glycosyltransferase B was used to effect the stereospecific synthesis of preparative amounts of human blood group B oligosaccharide antigen. This is the first example of the construction of a completely synthetic glycosyltransferase gene, and of its successful expression.

S32

Determination of Genuine Lewis Blood Group Types in Cancer Patients

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We previously reported that the frequency of individuals with Lewis-negative erythrocytes increased in cancer patients and that some cancer patients secreted not only Lewis antigens but also α(1,4) fucosyltransferase (Le enzyme) in their salivas although the phenotypes of their erythrocytes were typed as Lewis negative [1]. Recently, we have analysed the genomic structures of *Fuc-TIII* genes and found three missence mutations in Lewis-negatives' *Fuc-TIII* genes. Two of them (*G508A* and *T1067A*) found in the catalytic domain of the Le enzyme have been proved to be cause for the inactivation of the enzyme. We have also developed PCR-RFLP methods to detect easily those three mutations [2].

Twenty of the 66 (30.3%) patients with various cancers were typed as Lewis negative from their erythrocytes and they were divided into three groups based on the presence of Lewis antigens and Le enzyme in saliva: group I who had Lewis antigens and Le enzyme; group II who had only Le enzyme; group III who had neither Lewis antigens nor Le enzyme. The Lewis genotyping with the aid of PCR-RFLP methods demonstrated that all 14 patients from group I and II possess *Le* gene homozygously (*Le/Le*) or heterozygously (*Le/le*) whereas all six patients from group III do not possess *Le* gene and were *le/le* homozygotes. Therefore, the presence of salivary Le enzyme was proved to be completely consistent with that of the *Le* gene. The assay for the detection of salivary Le enzyme or the genotyping of the *Le* gene gives essential information on genuine Lewis blood group types in cancer patients.

1. S. Yazawa *et al.* (1988) *Jpn J Cancer Res* **79**: 538.
2. S. Nishihara *et al.* (1994) *J Biol Chem* **269**: 29271.

S33. ABERRANT GLYCOSYLATION CAUSING DISEASE PROGRESSION

Chair: Raymond Dwek

S33. 4.00pm

Studies on the Mechanism of Action of *N*-butyldeoxynojirimycin as an Inhibitor of HIV Replication

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The α-glucosidase inhibitor *N*-butyldeoxynojirimycin (NB-DNJ) is a potent inhibitor of HIV replication and syncytia formation *in vitro*, and is currently in clinical trials as an HIV therapeutic. However, the exact mechanism of action of NB-DNJ still remains to be established. In this study we examined the effect of NB-DNJ on the output of virus particles and their infectivity, with particular emphasis on viral entry.

Using a 24 h output assay, NB-DNJ was found to have only a small effect on virus output. The infectivity of virus particles released was, however, greatly reduced at concentrations of NB-DNJ higher than 0.5 mM. Using two independent entry-assays (based on either PCR or HIV-Cocal pseudotypes) the reduction in infectivity of virus produced in the presence of NB-DNJ was found to be due to an impairment of viral entry. No effect of NB-DNJ treatment was found on the affinity between gp120 and CD4, neither did NB-DNJ treatment affect the binding of virus particles to CD4-positive cells. These results conclusively show that a major mechanism of action of NB-DNJ as an inhibitor of HIV replication is inhibition of viral entry at the level of post-CD4 binding due to an effect on viral envelope components.

S33. 4.25pm

Mannose Corrects Underglycosylation in Carbohydrate Deficient Glycoprotein Syndrome Cells

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Carbohydrate Deficient Glycoprotein Syndrome (CDGS) is a multisystemic recessive metabolic disorder characterized by altered glycosylation of multiple proteins. The primary defect is unknown, but patients with Type 1 CDGS fail to add entire N-linked oligosaccharide chains to many proteins. We labelled the N-linked sugar chains of fibroblasts from 12 different patients with [2-^{3}H] mannose and found that 10 of them incorporated 3–15 times less label into glycoproteins and into various mannose intermediates than controls. The CDGS cells also made abnormally small lipid-linked oligosaccharide (LLO) precursors showing that decreased incorporation was not simply a labelling artifact. These results suggested that CDGS cannot utilize/produce sufficient mannose for glycoprotein synthesis. Simply adding 0.05–0.2 mg ml^{-1} D-mannose to the culture medium coordinately corrects both underglycosylation and the truncated size of the oligosaccharides. Increased glucose or adding L-mannose does not correct the defect. We have evidence that mannose enters fibroblasts through a process that is not inhibited by 0.5–5 mM glucose. Mannose entry into cells shows an apparent uptake of 18 μM in normal cells, and 35 μM in one CDGS patient but was unmeasurably high in another. Since plasma contains about 50 μM mannose, the results suggest that a putative transporter could be physiologically important for supplying mannose to cells. Moreover, a simple dietary supplement of mannose might benefit CDGS patients.

Supported by NIGMS 49096.

S33. 4.50pm

A Slowly Degrading Form of CFTR Located in the Surface Membranes of Wild Type and Mutant Cystic Fibrosis (CF) Airway Cells

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CFTR is the glycosylated product of the gene which is mutated in CF. Wild type CFTR is a transmembrane glycoprotein which localizes in the surface membrane where it serves as an apical Cl^- channel in epithelial cells. At least in some cases, CFTR of mutant cells also resides in the surface membrane [1]. Although the recycling of specific membrane glycoproteins between the surface membrane and the TGN has been examined, there is little information available on the other glycoproteins [2]. Surface localized CFTR was examined by pulse/chase experiments with immortalized CF (ΔF508) and non-CF (wt) airway epithelial cells using saturation labelling with [^{35}S]Met and a chase for 5 or 24 h. Total cell membranes [3] and whole surface membranes [1] were prepared and turnover ratios determined by phosphorimaging of the immunoreactive glycoprotein. Surface membrane localized CFTR was degraded more slowly than the total membrane CFTR. Moreover, DF508 CFTR in the surface membrane appeared stable as little degradation was observed even to 24 h whereas wt CFTR was degraded. This altered processing of CFTR may be linked to the pathogenesis of the disease and to the altered glycosylation which is characteristic of CF [4].

NIH RO1 DK 16859 and (AEM) KII DK 02077.

1) Harsch AD *et al.* (1992) *Chest* **101**: 58s–60s.
2) Kornfeld S (1992) *Annu Rev Biochem* **61**: 307–30.
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S33. 5.15pm

Gangliosides as Risk Factors in Atherosclerosis

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The ganglioside level in atherosclerotic lesions is considerably higher than in unaffected areas of the aorta, and atherosclerotic patients have increased sialic acid concentration in plasma. In this connection we studied the influence of gangliosides on the structure and functions of low-density lipoproteins (LDL) and on some factors involved in the formation of atherosclerotic plaques [1]. Preincubation of LDL with small amounts of gangliosides was found to cause structural changes of the LDL surface, to induce aggregation of LDL and to inhibit their binding to hepatic cells, thus possibly interfering with removal of cholesterol by the LDL-receptor mediated pathway. Furthermore, gangliosides were found to stimulate LDL uptake by and accumulation of cholesterol and triglycerides in macrophages leading ultimately to formation of foam cells. The ganglioside spectra of atherosclerotic intima cells were characterized by high levels of GD3, a marker of many fast growing cells and tissues. GD3 (but not other aorta gangliosides) induced rapid adhesion, spreading and aggregation of platelets, suggesting that intimal GD3 exposed into the lumen after endothelial injury may be responsible for platelet attachment to the damped vessel wall. In combination, our data suggest that high ganglioside levels may be a factor promoting processes involved in the development of atherosclerosis.

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S33. 5.35pm

Carbohydrate Composition of Serum Transferrin Isoforms from Patients with High Alcohol Consumption

E. Landberg[1], P. Påhlsson, A. Lundblad, A. Arnetorp[2] and J.-O. Jeppsson[2]
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Human transferrin is an iron transporting plasmaprotein, which is present in several isoforms in normal serum. By isoelectrical focusing these transferrin isoforms separate into several bands with different isoelectrical points (pIs). This separation is due to the number of sialic acid residues carried by the different transferrin isoforms. Transferrin has two potential glycosylation sites, both normally occupied by oligosaccharide chains. Two of the transferrin isoforms, called carbohydrate deficient transferrin (CDT), are specifically increased in patients with high alcohol consumption. The carbohydrate composition of the CDT isoforms have not yet been determined. In this study, five isoforms of transferrin were isolated from patients with high

alcohol consumption. Transferrin was purified by affinity chromatography and the isoforms were separated by high pressure liquid chromatography. N-linked glycans were released by N-glycosidase digestion, and were radioactively labelled by NaB^3H_4 reduction. The purified oligosaccharides were analysed by high-pH anion-exchange chromatography, and the carbohydrate composition of each individual transferrin isoform was determined. N-glycans found on transferrin isoforms with pI 5.2, 5.4 and 5.6 were in accordance with earlier defined structures. These isoforms are also present in serum from normal individuals. The CDT isoforms, which are especially increased in high alcohol consumers (pI 5.7 and 5.9), were found to lack one or both of their entire carbohydrate chains.

S33. 5.55pm

Detection and Analysis of Carbohydrate-based Disease 'Markers' in Human Plasma and Urine

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The recent growth of interest in the field of Glycobiology has been partially fuelled by the demonstrated importance of carbohydrates in the function and pharmacokinetics of glycoprotein therapeutics, as well as the potential of carbohydrates as diagnostic 'markers' of disease. The central role played by carbohydrates in many metabolic processes helps to explain why abnormal carbohydrate metabolism often results in detectable carbohydrate 'markers' that can be used to diagnose specific diseases. Carbohydrate 'markers' have been described for a number of human diseases including diabetes, osteoporosis, arthritis, liver disease, cancer, and a variety of inherited metabolic diseases. In this presentation we will describe a relatively simple method for routine analysis of carbohydrate-based disease 'markers' found in blood and urine. The method we will highlight, which is based on the separation of fluorophore-tagged carbohydrates by PAGE, is called fluorophore-assisted-carbohydrate-electrophoresis or FACE®. We will show how the FACE technique has been used to detect diagnostic carbohydrate 'markers' for the lysosomal storage diseases, osteoporosis, arthritis, alcoholism, and as a method for following the pharmacokinetics of carbohydrate-based drugs such as heparin.

S33 POSTERS

S33

Cultured Bovine Articular Chonducytes Synthesize and Secrete a High Molecular Weight Anionic Glycoconjugate (HMW AG) Whose Synthesis is Specifically Stimulated by TGF-β

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Department of Medicine, Queen's University, Kingston, Ontario, Canada K7L 3N6.

TGF-β is considered to be an important peptide in articular cartilage repair and osteoarthritis. Using gel filtration and DEAE-anion exchange chromatography in 8 M urea, we have isolated and identified a HMW AG in the media of cultures of bovine articular chondrocytes stimulated with TGF-β. The HMW AG, which eluted at the beginning of the proteoglycan peak from DEAE-anion exchange chromatography and was labelled primarily with ^3H from ^3H-glucosamine, was not readily observed in media of non-stimulated cultures. The HMW AG has an apparent M_r of approximately 540 000 on 2.5–10% SDS-PAGE, where it migrates as a single radiolabelled band. It stained blue with the 'Stains All' dye, but is not detected by standard protein stains, suggesting heavy glycosylation. Using anion exchange chromatography on a Mono Q FPLC column, it was further purified to a homogeneous product, without evidence of contaminating proteins, within the limits of the autoradiography and stain methods for the gels, and was not reduced by 2-mercaptoethanol even after extensive treatment. Amino-acid analysis indicated high aspartic and glutamic acid content. Specific enzymatic treatments showed that the HMW AG is susceptible to trypsin, it is heavily glycosylated with O-linked glycan chains, containing galactosamine, glucosamine and sialic acid and contains no glycosaminoglycan chains.

The HMW AG was also studied in cultures which had been serially subcultured to express the dedifferentiated phenotype. Both control and TGF-β-treated subcultures synthesized increased quantities of low M_r PGs. However, the HMW AG was readily detectable in the media of the TGF-β-treated subcultures but not in the media of the control subcultures, suggesting that the synthetic response of the HMW AG to the added TGF-β does not depend on the differentiation state of the chondrocytes.

S33

Identification of Core 2N-Acetylglucosaminyltransferase as a Diabetes and Hyperglycemia-Induced Gene

Y. Nishio, C. E. Warren, J. A. Buczek-Thomas, J. Rulfs, D. Koya, L. P. Aiello, E. P. Feener, T. B. Miller, Jr., *J. W. Dennis* and G. L. King
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Primary cardiac abnormalities have been frequently reported in patients with diabetes probably due to metabolic consequences of the disease. Approximately 2000 mRNA species from the heart of streptozotocin induced diabetic and control rats were compared by the mRNA differential display method, two of eight candidate clones thus isolated (DH1 and 13) were confirmed by Northern blot analysis. DH1 was predominantly expressed in the heart with an expression level 6.8 fold higher in the diabetic rats than in controls ($p < 0.001$). Insulin treatment significantly ($p < 0.001$) normalized the expression of DH1 in the hearts of diabetic rats. The expression in cardiomyocytes was regulated by insulin and glucose concentration of culture media. DH1 cDNA had a single open-reading frame with 85 and 92% amino acid homology to human and mouse UDP-GlcNAc:Gal1-3GalNAcαR β1-6 *N*-acetylglucosaminyltransferase (core 2 GlcNAc-T), a key enzyme determining the structure of O-linked glycosylation. Transient transfection of DH1 cDNA into Cos7 cells conferred core 2 GlcNAc-T enzyme activity. *In vivo*, core 2 GlcNAc-T activity was increased by 82% ($p < 0.05$) in diabetic hearts vs. controls, while the enzymes GlcNAc-TI and GlcNAc-TV responsible for N-linked glycosylation were unchanged. These results suggest that core 2 GlcNAc-T is specifically induced in the heart by diabetes or

hyperglycemia. The induction of this enzyme may be responsible for the increase in the deposition of glycoconjugates and the abnormal functions found in the hearts of diabetic rats.

S33

The Effects of TGF-β And IGF-I on Chondroitin Sulfate Synthesis by Bovine Chondrocytes Utilizing a β-D-xyloside Acceptor

E. Wong and T. Anastassiades
Department of Medicine and Biochemistry, Queen's University, Kingston, Ontario, K7L 3N6, Canada.

Transforming growth factor-beta (TGF-β1) and insulin-like growth factor-I (IFG-I) are considered to be important in articular cartilage repair and osteoarthritis. In the present study we concentrated on the effects of these growth factors and their combination on the synthesis of chondroitin sulfate proteoglycan (CSPG). We have used a β-D-xyloside, which competes for the endogenous galactose acceptors on the core protein of CSPG, to study the post-translational modification of CSPG in the presence or absence of these growth factors. Monolayer cultures of bovine articular chondrocytes treated without or with TGF-β (5 ng ml^{-1}), or IGF-I (150 ng ml^{-1}), or TGF-β + IGF-I were incubated for 2 days with [^{3}H]glucosamine and [^{35}S]SO_4^{2-} either in the presence or absence of p-nitrophenyl-β-D-xyloside (0.6 mM). Incorporation of radioactivity into the glycosaminoglycan (GAG) chains was quantified by cetylpyridinium chloride precipitation of the GAG, following papain digestion and separation by cellulose acetate electrophoresis. Characterization of the CSPG and the β-D-xyloside-initiated free GAG chains was done by using anion exchange (DEAE) and gel filtration (Sepharose CL2B and CL6B) chromatography. In the absence of β-D-xyloside, the addition of TGF-β, IGF-I and their combination increased the incorporation of [^{35}S]SO_4^{2-} into the CS by about 2.5, 1.8 and 4.4 fold, respectively. Characterization of the CSPG showed that the hydrodynamic size of the CSPG had increased upon the addition of these growth factors, furthermore their combination had further increased the hydrodynamic size of the CSPG. In the presence of β-D-xyloside, most of the [^{3}H] and [^{35}S] were incorporated into the β-D-xyloside-initiated free GAG chains in both growth factor-treated and untreated cultures. The addition of TGF-β and its combination with IGF-I, but not IGF-I alone, had increased the incorporation of [^{35}S]SO_4^{2-} into the free GAG chains by about 1.4 and 1.3 fold, respectively. These free GAG chains generated were characterized by Sepharose CL6B, and upon the addition of TGF-β, or its combination with IGF-I, the hydrodynamic sizes have been increased to the same extent: however, the addition of IGF-I caused no significant change in the hydrodynamic size of the free GAG chains. These results suggest that the stimulation of CSPG synthesis by TGF-β is mediated, at least in part, by an increase in GAG chain length while stimulation of CSPG synthesis by IGF-I may be mediated by an increase in the number of GAG chains.

SATURDAY 26 AUGUST, MORNING

PLENARY LECTURES

S34. 8.30am

Treatment of Gaucher's Disease with an Enzyme Inhibitor

N. S. Radin[1], A. Abe[1], J. A. Shayman[1], B. Ganem[2]
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[2]*Department of Chemistry, Cornell University, Ithaca, USA.*

At present, this genetic disorder in glucosylceramide (GlcCer) glucosidase is treated by injecting patients with a modified form of the normal human enzyme. The exogenous enzyme supplements the weakly active mutant form of the enzyme, resulting in gradual reduction in the amount of stored GlcCer. This approach is very expensive and must be used throughout the patient's life. An alternative approach, the use of inhibitors of GlcCer synthase, would slow synthesis of GlcCer and allow the mutant enzyme to gradually hydrolyse accumulated GlcCer.

The inhibitors appear to be nontoxic and rapidly effective in mice, fish, and cultured cells. The compounds resemble GlcCer in structure but contain a 3° cyclic amine in place of the glucose moiety; most of the compounds studied contain a phenyl ring in place of the long alkenyl chain of sphingosine. The inhibitor studied in depth (PDMP = D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol) produced a 35% decrease in the level of kidney GlcCer of normal mice within 5 h. Mice injected daily with PDMP for 12 days exhibited no signs of toxicity except for a slight decrease in kidney size. Differential cell counts were normal and the mice appeared to be healthy for at least 6 months. The fish grown as embryos in PDMP-containing water hatched normally and showed normal physiological behaviour despite a marked absence of glycosphingolipids. Cultured cells grown in PDMP were greatly depleted of their GlcCer content yet grew normally after exposure to normal medium.

Versions of PDMP with longer fatty acyl chains, a 5-membered ring (pyrrolidine) instead of morpholine, and a sphingosine chain instead of a phenyl group were much more active against the synthase, depleting cultured cells noticeably within 24 h at 0.2 μM.

S35. 9.00am

Polylactosamine Sugars Induce Proliferation and IL-10 Production from B Cells of Th2-type ($CD4^+$ T Cells) Mice

D. A. Harn[1], A. Horauf[2] and V. Palanivel[1]
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In murine *Schistosomiasis mansoni*, schistosome-eggs or saline soluble egg antigens (SEA) generate Th-2 type (IL-4, IL-5, IL-10) $CD4^+$ T cell responses to schistosome as well as unrelated antigens. Chemical alteration or removal of glycans

on egg antigens reduces lymphocyte stimulatory activity. Lacto-n-fucopentaose III (LNFPIII) and lacto-N-neotetraose (LNT) are found on egg antigens and, in purified form, drive proliferation of B cells from naive and/or infected Th2 type mice but not Th1-type mice. LNFPIII induces B-cells from Th2-type mice to produce IL-10, a Th1 downregulatory cytokine. Th2-type mice also have increases in IL-10 producing B-1 (B220 + CD5$^+$) cells after infection or injection of sugars. We also examined the role of polylactosamine sugars in leishmaniasis, an intramacrophage parasite where Balb/c (Th-2 type) mice have progressive infection and die and Balb/c Xid mice (Th-1 type) are resistant. Xid mice have a complete lack of B-1 cells and an absence of antibodies to polysaccharide antigens. Splenocytes from Balb/c infected mice had responses to *L. major* lysate or lacto-N-fucopentaose III of 32695 and 12787 cpm respectively, compared to 6346 and 146 cpm in Balb.Xid. Levels of IL-10 were 159 vs 0 pg ml^{-1} in Balb/C vs Balb.Xid (stimulated with lysate) and 597 vs 0 pg ml^{-1} (stimulated with LNFPIII). Levels of splenic B220$^+$ cells and peritoneal B-1 cells were 75% and 31%, respectively, in Balb/c compared to 55% and 1% in Balb.Xid. These results imply that early expansion of B/B-1 cells by polylactosamine sugars and associated synthesis of IL-10 may be initial driving forces leading to Th2-type CD4$^+$ T cell dominance in a number of diseases.

S34. TRANSMEMBRANE SIGNALLING CONTROL BY SPHINGOLIPIDS

Chairs: Sarah Spiegel, Richard Kolesnick

S34. 9.50am

Introduction

S34. 9.55am

Ceramide: A Stress Responder and Tumour Suppressor Lipid

Yusuf A. Hannun, Lina M. Obeid
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Studies from our laboratory have resulted in the identification of the sphingomyelin cycle as a novel sphingolipid-mediated pathway of signal transduction and cell regulation. Multiple lines of evidence are beginning to implicate ceramide, the product of sphingomyelin hydrolysis, as a novel second messenger with important cellular activities. Studies on the mechanisms of regulation of the sphingomyelin cycle have resulted in identification of a number of cytokines and extracellular agents (such as tumour necrosis factor α) which induce activation of neutral sphingomyelinases resulting in the formation of ceramide. In turn, ceramide functions as an important mediator of growth inhibition, cell differentiation, regulation of gene expression, and induction of apoptosis. Studies on the mechanism of action of ceramide have resulted in the identification of a ceramide activated protein phosphatase (CAPP) which is a serine/threonine protein phosphatase of the PP2A family. This phosphatase is activated by ceramide *in vitro* and in cells, and evidence points to the involvement of this phosphatase in mediating cellular activities of ceramide such as down regulation of *c-myc* and induction of apoptosis.

Other recent studies are also beginning to implicate ceramide as a mediator of cell cycle arrest. Ceramide-induced G0/G1 cell cycle arrest involves activation of the retinoblastoma gene product (Rb), in a mechanism distinct from induction of apoptosis.

These studies on the mechanism of regulation of sphingomyelin hydrolysis and the determination of cellular activities of ceramide point to a major role for ceramide as a responder to extra and intracellular inducers of stress/injury resulting in growth suppression, cell differentiation, or apoptosis.

S34. 10.20am

Sphingolipid Metabolites, Sphingosine and Sphingosine-1-Phosphate, Members of a New Class of Second Messengers

Sarah Spiegel, Ana Olivera and Fang Wang
Department of Biochemistry and Molecular Biology, Georgetown University Medical Center, Washington DC, USA.

The potent mitogens, PDGF and foetal calf serum, rapidly and transiently increased levels of sphingosine and sphingosine-1-phosphate in Swiss 3T3 fibroblasts. Similarly, the B subunit of cholera toxin, which binds specifically to ganglioside GM1 and stimulates DNA synthesis, also induced a significant increase in sphingosine-1-phosphate. PDGF, serum, and the B subunit also transiently activated cytosolic sphingosine kinase activity. In contrast, EGF had no effects on sphingosine, sphingosine-1-phosphate, or sphingosine kinase. DL-*threo*-dihydrosphingosine, a competitive inhibitor of sphingosine kinase, reduced DNA synthesis induced by PDGF, serum, and the B subunit but did not abrogate cellular proliferation induced by EGF. Our results indicate that increased DNA synthesis and cell division induced by sphingolipids metabolites may result from activation of the transcription factor activator protein-1 (AP-1). Sphingosine and sphingosine-1-phosphate are members of a new class of lipid second messengers: they elicit diverse cellular responses; their turnover is extremely rapid; their levels in cells are very low and increase rapidly and transiently in response to growth factors; they can release calcium from internal sources via an InsP$_3$-independent manner, and stimulate phospholipase D leading to increase in the level of phosphatidic acid, two important events in the control of cellular proliferation; finally, the activation of AP-1, which is one of the transcription factors activated by the Ras-MAPK pathway, is the first link between the effects of these sphingolipids metabolites on cellular proliferation and gene expression.

S34. 10.45am

Ceramide-Activated Protein Kinase is a Raf Kinase

Richard Kolesnick
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Ceramide-activated protein kinase (CAPK) is a proline-directed serine/threonine kinase central to ceramide-mediated signal transduction across the plasma membrane. CAPK has previously been shown to recognize the minimal substrate sequence -T-L-P-. Since prior investigations from this laboratory suggested that ceramide-mediated signal transmission through the cytosol involved activation of MAP kinases, we investigated whether CAPK might activate Raf-1. We show ceramide-activated protein kinase is a Raf kinase. *In vitro*, CAPK phosphorylated Raf-1 on Thr^{269}, which is contained within a -T-L-P- motif, increasing its activity toward MEK. In intact HL-60 cells, TNF and ceramide analogues induced CAPK to complex tightly with and phosphorylate Raf-1, increasing Raf-1 activity. These investigations identify CAPK as a link between the TNF receptor and Raf-1.

S34. 11.10am

Sphingomyelinases in TNF Signalling

M. Krönke
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Ceramide produced by sphingomyelinases (SMase) has been recognized as an important second messenger in growth factor receptor signalling. Tumour necrosis factor (TNF) through binding to the 55 kDa TNF receptor (TNF-R55) rapidly activates two distinct types of SMase, a membrane-associated neutral (N-)SMase, and an endosomal acid (A-)SMase. N-SMase and A-SMase are activated independently by different cytoplasmic domains of TNF-R55. Each type of SMase specifically couples to select pathways of TNF signalling. Ceramide generated by N-SMase directs the activation of proline-directed serine/threonine protein kinase(s), Raf-1 kinase, and phospholipase A_2. In contrast, A-SMase is secondary to the action of a phosphatidylcholine-specific phospholipase C (PC-PLC) and triggers the activation of NF-κB. No apparent crosstalk was detected between N-SMase and A-SMase pathways, indicating that ceramide action depends on the topology of its production. These results suggest that N-SMase and A-SMase control important yet dissociable and non-overlapping pathways of TNF receptor signal transduction.

S34. 11.35am

Sphingosine-1-Phosphate Acts as an Intercellular but Not Intracellular Messenger in Platelet Activation and Cell Motility Inhibition

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Sphingosine-1-phosphate (Sph-1-P) has been recognized as one of the bioactive sphingolipids, which plays an intracellular messenger role in PDGF or serum factor-dependent 3T3 fibroblast growth. We have recently reported that Sph-1-P inhibits the chemotactic and haptotactic motility and invasion (through matrigel) of various cancer cells at a very low, nM order, concentration with Boyden chamber *in vitro* assay system [1, 2]. More recently, we have also shown that exogenously-added Sph-1-P induces platelet shape changes through Ca^{2+} mobilization in the cells and that upon agonist (thrombin or collagen) administration, Sph-1-P stored in platelets is released into the medium and amplifies platelet aggregation reaction further. In this study, in order to examine whether Sph-1-P acts as an intracellular or intercellular messenger towards these phenomena, we synthesized immobilized Sph-1-P on controlled pore glass beads through amide linkage with a newly introduced Sph-1-P derivative possessing ω-carboxyl group. Using immobilized Sph-1-P, we showed that Sph-1-P has a receptor on the cell surface and acts as an intercellular messenger towards platelet activation or cancer cell motility inhibition, which is quite different from the intracellular messenger roles of the molecule played in growth regulation of 3T3 fibroblasts. Our results suggest that the regulation of not only Sph-1-P synthesis by Sph kinase inside the cells but also release of Sph-1-P from the cells might be a very important step in many cell functions.

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2. Sadahira Y, Zheng M, Ruan F, Hakomori S, Igarashi Y (1994) *FEBS Lett* **340**: 99–103.
3. Yatomi Y, Ruan F, Hakomori S, Igarashi Y (1995) *Blood* in press.

S34. 11.50am

The Difference of Signal Transduction Between Ceramide and Sphingosine-Induced Apoptosis in HL-60 Cells

T. Okazaki[1], H. Sawai[2], M. Tashima[2], H. Sawada[2] and N. Domae[1]
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[2] *First Division, Faculty of Medicine, Kyoto University, Kyoto, Japan.*

Ceramide has emerged as a lipid mediator for differentiation and apoptosis in human leukaemic HL-60 cells. Sphingosine consisting of the backbone structure of ceramide was reported as a lipid mediator for proliferation in rat fibroblasts. However, in HL-60 cells sphingosine seems to function as a inhibitory molecule for protein kinase C. When we studied the effects of sphingosine on HL-60 cell apoptosis, cell growth was inhibited and DNA fragmentation was seen. Previously we showed that ceramide induced the increase of the c-jun message and that AP-1 plays a crucial role in the induction of apoptosis in HL-60 cells. This increase of c-jun mRNA was inhibited by the addition of H-7 less than by that of staurosporine. Sphingosine also increased the c-jun message but it was inhibited by the addition of H-7 more than by staurosporine. These results suggested that sphingosine induced the increase of c-jun mRNA in a different manner from that for ceramide. D-Threo-dihydrosphingosine, which is supposed to be an inhibitor of sphingosine kinase, did not affect the increase of c-jun mRNA by sphingosine, suggesting that sphingosine was not converted to sphingosine-1-phosphate. Taken together, these suggest that sphingosine induces apoptosis in HL-60 cells by using a different pathway of signal transduction from ceramide without converting to sphingosine-1-phosphate.

S34 POSTERS

S34

Effect of Sphingolipids and Glycosphingolipids (GSLs) on Interleukin-1-Dependent E-Selectin Expression in Human Umbilical Vein Endothelial Cells (HUVEC)

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E-selectin-dependent adhesion followed by transendothelial migration of leukocytes and tumour cells is of central importance for understanding the molecular mechanism of initiation of inflammation and tumour metastasis. E-selectin is synthesized *de novo* in endothelial cells (ECs) and expressed at the EC surface following stimulation with IL-1 or TNF-α. The mechanism therefore involves transmembrane signalling pathways through IL-1 or TNF-α receptors, leading to activation of transcription factors required for E-selectin synthesis. It is well known that NF-κB activation is essential for E-selectin expression, but the regulatory mechanism is not fully understood. We investigated the effect of several sphingolipids and GSLs on E-selectin expression in HUVEC using enzyme-linked immunosorbent assay and flow cytometry. C2-ceramide enhanced IL-1-induced E-selectin expression two- to three-fold, but did not induce the expression by itself. Bacterial sphingomyelinase mimicked this effect of C2-ceramide. Sphingosine (Sph), *N,N*-dimethyl-Sph-1-phosphate, LacCer, and GM3 ganglioside had no effect on E-selectin expression. Electrophoretic mobility shift assay revealed that C2-ceramide activated NF-κB in HUVEC and enhanced IL-1-dependent activation, suggesting that its effect is through NF-κB at the transcriptional level. These and other findings suggest that some sphingo(glyco)lipids may play roles in regulation of IL-1-dependent E-selectin expression in HUVEC. Further systematic studies using other GSLs and their derivatives are in progress.

Supported in part by NIH OIG CA-42505 and by funds from The Biomembrane Institute.

S34

Sphingosine as a Messenger in Signalling Apoptosis

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Apoptosis in a variety of human cancer cells can be induced by treatment with sphingosine (SPN) or its methylated derivative *N,N*-dimethylsphingosine (DMS) as analysed by Flow Cytometry, morphology, and DNA fragmentation. Ceramide analogues, however, failed to induce apoptosis under the same experimental conditions, and, although apoptosis was seen with these analogues in the absence of serum, SPN and DMS were much stronger inducers. The apoptosis caused by SPN and DMS was completely blocked by protease inhibitors. In the study of apoptosis caused by signal transduction through *fas* and *tnf* receptors, the sphingomyelin pathway has been implicated to be involved in the program of cell death. Our evidence supports the suggestion that sphingosine, rather than or in addition to ceramide, is a major messenger in this pathway as part of the mechanism of apoptosis.

S34

Sphingosine-1-Phosphate Release from Platelets into Serum During Blood Clotting. Quantitative Measurement of Sphingosine-1-Phosphate by Acylation with Radioactive Acetic Anhydride

Y. Yatomi, F. Ruan, H. Ohta, R. J. Welch, S. Hakomori and Y. Igarashi
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Departments of Pathobiology and Microbiology, University of Washington, Seattle, WA, USA.

Sphingosine-1-phosphate (Sph-1-P) has been shown to be involved in diverse cellular processes. Although the physiological roles of this bioactive sphingolipid are strongly suggested, there have been few studies reporting the presence of Sph-1-P in cells or body fluids under physiological conditions. We describe here the development of a simple and sensitive method for quantitative measurement of Sph-1-P to assess its physiological functions. This assay is based on the quantitative conversion of Sph-1-P to *N*-[^{3}H]acetylated Sph-1-P by *N*-acylation with [^{3}H]acetic anhydride. Compared with other blood cells, platelets were found to possess Sph-1-P abundantly; the amount of Sph-1-P in platelets was 141 ± 4 pmol per 10^8 cells (mean $\pm$ SD, $n = 3$) and about four times higher than that of sphingosine. Furthermore, plasma and serum were found to contain 197 ± 119 pmol ml^{-1} and 405 ± 102 pmol ml^{-1} of Sph-1-P (mean $\pm$ SD, $n = 6$), respectively. It is most likely that the source of discharged Sph-1-P during blood clotting is platelets because Sph-1-P is abundantly stored in platelets and they release their contents during clot formation. To support this, platelets were found to release part of their stored Sph-1-P into the medium upon challenge with the physiological agonist, thrombin. Since Sph-1-P reportedly activates platelets, stimulates fibroblast proliferation, and regulates cell motility and tumour cell invasiveness, Sph-1-P may play important roles in thrombosis, haemostasis, atherosclerosis, wound healing, and cancer metastasis.

S35. GLYCOBIOLOGY OF IMMUNOCYTES AND IMMUNE RESPONSES

Chairs: Toshi Osawa, Ten Feizi

S35. 9.50am

Carbohydrate-Directed Effector Functions of Natural Killer Cells

T. Feizi
The MRC Glycosciences Laboratory, Northwick Park Hospital, Harrow, UK.

Natural killer (NK) cells are lymphocytes which have roles in innate immunity rather than the acquired immunity conferred by immunization. These cells are able to kill certain tumour cells and cells infected with viruses but not others. Studies of the rat NK system have shown that cancer cells which are NK-susceptible have on their surface carbohydrate ligands recognized by a lectin, NKR-P1, at the killer cell surface. The oligosaccharide ligands for NKR-P1 include members of the blood group and ganglio families as well as glycosaminoglycans. The key elements of the most active carbohydrate ligands, the glycosaminoglycans, are small, consisting of only one or two monosaccharides carrying a negative charge, making it potentially easy to synthesize drugs based on these ligands. If such ligands are converted to neoglycolipids and embedded as clusters on the surface of cancer cells that are normally NK-resistant, they become vulnerable and are destroyed. This new principle is potentially a powerful means of harnessing the body's own NK cells to purge the body of undesirable cells such as cancer cells and those infected with viruses [1].

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S35. 10.15am

Galectin-1-Induces Apoptosis of T Cells

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Galectin-1 is a 14.5 kDa member of the family of β-galactoside binding lectins. Galectins are found in a variety of species from invertebrates to humans and have been proposed to have growth-regulatory and immunomodulatory activities. We have recently shown that galectin-1 is expressed by stromal cells in thymus and lymph nodes, sites at which programmed cell death of lymphocytes occurs. Galectin-1 appears to participate in this process, as binding of galectin-1 to human thymocytes and activated T cells induced apoptosis of the cells *in vitro*. To identify candidate counter-receptors for galectin-1 which may be involved in signalling, we examined a panel of antibodies against T cell surface glycoproteins for the ability to block galectin-1 binding. Antibodies to the T cell glycoproteins CD43 and CD45 inhibited binding, while antibodies to other T cell surface molecules had no inhibitory effect. We examined the role of CD45, a tyrosine phosphatase, in galectin-1-induced apoptosis. HPB.ALL cells, which express CD45, underwent apoptosis upon binding galectin-1. The mutant HPB.450 cell line, which lacks CD45, bound galectin-1, but did not undergo apoptosis. N-glycans on T cell surface molecules appeared to participate in galectin-1-induced death, since treatment of cells with swainsonine reduced galectin-1 binding and abrogated the apoptotic effect of galectin-1. We propose that galectin-1 participates in the clonal deletion of T cells, by binding to specific oligosaccharide sequences on CD45.

S35. 10.40am

Immunosuppression by Synthetic and Tumour Gangliosides

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New chemical synthetic methods have permitted the synthesis of a spectrum of novel glycosphingolipid molecular species of potential biological significance. For example, dialkane G_{M3}, which has a branched alkane in place of ceramide, markedly inhibits the human lymphoproliferative response *in vitro* ($ID_{90} < 7\ \mu M$) and is five-fold more active than natural G_{M3}. Studies of other modified molecular species of G_{M3} and G_{M4} show that structural variations among gangliosides significantly influence their immunosuppressive activity *in vitro*. These studies have examined: (i) fatty acyl chain length; (ii) hydroxylation of fatty acyl group; (iii) substitution of an S-glycosidic bond for an O-glycosidic bond in the sialic acid ketosidic linkage; and (iv) modifications of the sialic acid group (KDN G_{M3} and G_{M4}, and 8-epi-G_{M3}).

To further assess the inhibitory effect of gangliosides on the cellular immune response *in vivo*, we have developed a murine model. Both neuroblastoma tumour gangliosides and certain synthetic gangliosides (particularly dialkane G_{M3}) are potent inhibitors; 10 nmol of these molecules injected subcutaneously together with an allogeneic cell challenge causing a marked inhibition of the cellular immune response in the draining popliteal lymph node. These findings strongly suggest an immunosuppressive role of tumour gangliosides *in vivo*, and a therapeutic potential of selected synthetic gangliosides.

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S35. 10.55am

Unusual Uniformity of the N-Linked Oligosaccharides Associated with Human HLA Class I Glycoproteins

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Allotypes of human histocompatibility class I glycoproteins possess a single conserved N-linked oligosaccharide addition site at position 86 of the heavy chain, close to the antigen-bind-

ing site. We characterized the N-linked oligosaccharides of human HLA class I allotypes expressed by Epstein Barr-virus-transformed B lymphocytes. The oligosaccharides were almost exclusively di-sialylated. Comparison of the size and relative abundance of oligosaccharides associated with nine class I allotypes revealed a similar and very restricted set of structures. Allotypes encoded by the HLA-A and -B loci contain two predominant structures; preliminary analysis of HLA-C-associated oligosaccharides indicates the presence of two additional structures. Characterization of the predominant oligosaccharides confirmed that identical structures were present on different allotypes. The uniformity of oligosaccharide structure associated with different HLA-A-A,B,C products and the relative lack of heterogeneity for any given allotype are unusual features for a mammalian glycoprotein. Particularly striking is that such conservation in oligosaccharide structure occurs at an addition site close to the major regions of amino acid sequence variation. The contribution of N-linked oligosaccharides to class I function remains unclear, although a role for oligosaccharide in the recognition of class I molecules by mouse natural killer cells has been suggested. However, we observed no correlation between the oligosaccharide structures present on class I allotypes and their differential recognition by human natural killer cells.

S35. 11.10am

Inhibition of Target Cell Glycosylation by Tunicamycin Abrogates Target Cells Lysis by Allospecific Cytotoxic T Lymphocytes

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P815 (H-2[d]) or EL-4 cells (H-2[b]) were treated for 24 hours with either tunicamycin (TM), swainsonine (SW), deoxynojiromycin (DNM) or deoxymannonojiromycin (DMM). All treatments significantly altered cell surface glycosylation as measured by FACS analysis of PHA-L and Con A binding, but did not inhibit surface localization of MHC I (Mab 34-5-8S). Treated and control cells were assessed for their ability to serve as targets in microcytotoxicity assays and as competitors in cold target inhibition assays using alloreactive CTL lines. While treatment with SW, DNM or DMM resulted in modest two to four fold reductions in the lysis of P815, treatment with TM abrogated both target cell recognition and lysis. EL-4 lysis was uneffected by SW, DNM and DMM; however, TM treatment again caused significant resistance to lysis. Thus, N-linked glycosylation, but not cell surface complex carbohydrates, seems to be required for target cell recognition and lysis by alloreactive effectors. Treatment with TM at levels 10 times higher than those used in this study (0.5 μg ml^{-1}) does not affect target cell recognition or lysis by lymphokine activated killer cells [1]. Further non-glycosylated MHC I is functional in antigen presentation. Therefore, the failure of alloreactive CTL to recognize the lyse TM treated targets probably results from the modification or loss of one or more ancillary adhesion or signalling molecules from the target cell surface due to the early blockade of N-linked glycosylation by TM. Experiments are currently underway to identify this molecule(s).

1. Mehta *et al*. (1994) *Cell Immunol* **155**: 95–110.
2. Miyazaki *et al*. (1986) *J Exp Med* **163**: 856–71.

S35. 11.25am

Removal of Human Anti-Pig Antibodies through Plasma Exchange/Immunoadsorption and *In Vitro* Neutralization with Soluble Oligosaccharides

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Background: Pig-to-man is a discordant species combination by definition leading to a transplanted organ being hyperacutely rejected. This rejection is believed to be initiated by the presence of preformed natural antibodies present in man against pig tissues. The targets for the antibodies have during the last few years been shown to be of a carbohydrate nature and the most important epitope seems to be the Galα1-3Gal structure. The first prerequisite for a transplantation is the removal of these antibodies.

Present study: We have studied the anti-pig antibodies in patients treated with plasmapheresis/immunoadsorption by lymphocytotoxicity, haemagglutination, flow cytometry and endothelial cell ELISA. We have also investigated the effect on titres of neutralization of antibodies with soluble di- and trisaccharides with terminal Galα1-3Gal structure. The titres of antibodies can be reduced to levels comparable to those where ABO-incompatible transplantations are performed. The return of the antibodies seems to occur in 1–2 weeks, sometimes to higher levels than before treatment.

In addition, results from a clinical trial with '*ex vivo*' connection of a pig kidney to the circulation of a human dialysis patient, pretreated with plasma exchange, will be presented.

Conclusion: Reduction of anti-pig antibodies to levels where a transplantation may be performed seems to be achieved by a series of plasma exchanges. Combination of plasma exchange and neutralization of remaining antibodies with soluble oligosaccharides may be the best pretreatment. Since the antibodies return in a few weeks, further exchange treatments may be necessary while waiting for accommodation.

S35. 11.40am

Studies on a Novel Murine Fibrogenic Lymphokine, Fibrosin

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Tissue fibrosis is a complication of several chronic inflammatory diseases. In an attempt to study the link between inflammation and fibrosis, we recently identified and cloned the cDNA of a novel fibrogenic lymphokine, fibrosin. Fibrosin is a heparin binding glycoprotein and is a potent fibroblast mitogen. It is produced by the CD4+ve, TH2 subclass of murine lymphocytes. For purposes of cloning, we used a cDNA library produced from the mRNA of activated T lymphocyte line CDC25, which produces fibrosin. By heterologous expression in Cos-7 cells of pools of CDC25 and cDNA library and screening for the biological activity in the conditioned medium, we isolated a cDNA clone. The culture supernatant of cells transfected with this clone stimulate fibroblast growth at a dilution of 1:20,000. This cDNA clone has an open reading frame of 216 nucleotides and shows no significant homology

with nucleotide sequences archived in the Genbank database. We now report that a synthetic peptide constructed from the deduced amino acid sequence is biologically active at picomolar concentrations. Other activities of this novel lymphokine are also being explored.

Initial work concerning the cloning of fibrosin was performed in collaboration with Dr. David J. Wyler at New England Medical Center, Boston, MA.

S35. 11.55am

Glycosphingolipids of Human Umbilical Vein Endothelial Cells

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Endothelial cells play an important role in the regulation of blood coagulation, inflammation and immune response. Activated endothelial cells express selectins (ELAM-1, P-selectin) which mediate cell-cell adhesion towards leukocytes via specific carbohydrate ligands. Although cell membrane proteins of endothelial cells have been the subject of many investigations, only little information is available about their lipid bound oligosaccharides.

Large production of human umbilical vein endothelial cells (HUVECs) was performed in a 2 l bioreactor by use of microcarriers as support for anchorage dependent growing cells. About 5×10^9 HUVECs were produced in medium supplemented with 20% (v/v) autologous human serum. Glycosphingolipids were isolated by standard procedures and their structures were determined by FAB-MS combined with TLC immunochemical techniques using carbohydrate specific antibodies. Beside the main neutral glycosphingolipids LacCer, $GbOse_3Cer$, $GbOse_4Cer$ and $nLcOse_4Cer$, small amounts of Le^x and trace quantities of Le^a positive bands were detected. The major gangliosides found were G_{M3}(Neu5Ac) and IV^3-Neu5Ac-$nLcOse_4Cer$, followed by IV^6Neu5Ac-$nLcOse_4Cer$ and small amounts of VI^3Neu5Ac-$nLcOse_6Cer$. Combined neuraminidase treatment with anti-Le^x immunostain revealed a few highly polar gangliosides with sialyl Le^x epitopes, expressed only at very low levels.

This approach, i.e. the large scale production of HUVECs on microcarriers in bioreactors, provides the basis of further investigation of glycosphingolipid expression on HUVECs in response to inflammatory agents.

S35 POSTERS

S35

Gangliosides of Porcine Aorta with Possible Relevance in Xenotransplantation

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When organs are transplanted between discordant species the graft undergoes hyperacute rejection. This depends on xenoreactive natural antibodies in the recipient, directed towards antigens located on endothelial cells of the transplanted organ. One major target for these antibodies is Galα1,3Gal-β1,4GlcNAcβ1,3Galβ1,4Glcβ1,1Ceramide but this is probably not the only one even among the neutral glycolipids. Among the acidic glycolipids binding of human antibodies have so far been detected to sulfatides [1] and screening against other acidic glycolipids is in progress.

We have prepared and characterized gangliosides from pig aorta (the non-acid glycosphingolipids have been described [2]). The fractions were separated on an open DEAE Sepharose column and pooled according to TLC migration. These fractions have been analysed by antibody overlay on HPTLC plates. Human AB serum shows binding to several bands. The fractions were then further subfractioned by HPLC on a silicic acid column and pooled into subfractions as above. The HPLC separated fractions have been analysed by FAB-MS as native compounds and evaluation of this work is in progress.

Porcine aorta ganglioside fractions will be used to screen for antibody binding in patient sera after pig kidney *ex vivo* perfusion.

1. Holgersson J, Cairns TDH, Karlsson EC, *et al.* (1992) *Transplant Proc* **24**: s605–8.

2. Hallberg EC, Holgersson J, Jovall P-Å, Samuelsson BE. Glycosphingolipid expression in pig aorta—possible target antigens for human natural antibodies. Submitted.

S35

The Oligosaccharides of the Japanese Cedar (*Cryptomeria japonica*) Pollen Allergen, *Cry j* I: Their Structures and Contribution to Antigenicities

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Cry j I, a major allergenic glycoprotein of Japanese cedar (sugi), is the most common pollen allergen in Japan. In this study, we elucidated the structures of the major N-linked oligosaccharides of *Cry j* I. After enzymatic release of N-linked oligosaccharides and subsequent pyridylamination, four major fractions (A–D) were obtained by HPLC. Two-dimensional sugar mapping analyses and 400 MHz ^{1}H-NMR spectroscopy showed that they are a series of biantennary complex type oligosaccharides that share the common fucose/xylose-containing core. The structure of oligosaccharide B was suggested to be a new structure containing a unique branch of fucosyl glucosamine. The oligosaccharide structures were in good agreement with our previous results of the carbohydrate compositions and lectin binding patterns of *Cry j* I [1].

Rabbit polyclonal anti-*Cry j* I IgG antibodies were found to be highly cross-reactive with plant glycoproteins having fucose/xylose-containing oligosaccharides by immunoblotting and ELISA, and the cross-reactivities were completely eliminated by chemical deglycosylation of the glycoproteins. ELISA inhibition showed that the majority of rabbit anti-*Cry j* I IgG antibodies recognized oligosaccharides on *Cry j* I and on these

plant glycoproteins. Allergic human IgE showed little cross-reactivities with these glycoproteins, and the carbohydrate epitopes were found to be only partially involved in the binding of specific IgE antibodies.

1. Hijikata A *et al.* (1994) *Int Arch Allergy Immunol* **105**: 198–202.

S35

9-O-Acetylated Sialic Acids on CD4 Positive Murine T Lymphocytes

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Terminal sialic acids on cell surface glycoconjugates can be 9-*O*-acetylated. Reports on their distribution in mammalian cells have been sporadic. In this study, we exploit the binding activity of an unique probe for 9-*O*-acetylated sialic acids, a recombinant soluble form of influenza C virus haemagglutinin-esterase, with the C-terminal transmembrane and cytoplasmic domains replaced by the Fc portion of human IgG and treated with diisopropylfluorophosphate to inactivate the esterase activity. Distribution of 9-*O*-acetylation on normal B10A mouse thymus, lymph nodes and spleen has been assayed with this probe by flow cytometry and immunohistochemistry, using appropriately conjugated secondary antibodies to detect binding. Of total thymocytes, 15 to 20% are *O*-acetylated and the majority of these are medullary thymocytes. Low levels of *O*-acetylation are seen in double negative and double positive cells and high levels in CD4 single positive (SP) cells. *O*-acetylation is present on 40% of CD4 SP cells and 8% of CD8 SP cells. Of all the *O*-acetylated cells, 60% are CD69 positive and 83% are CD5 (high) expressors indicating the association of *O*-acetylation with early T cell activation markers. While low levels of *O*-acetylation occur in all peripheral lymphocytes, a distinctly high degree of *O*-acetylation is present on 80–85% of CD4 cells. In contrast, only 8 to 12% of CD8 T lymphocytes and 3–5% of B lymphocytes show such a high level of *O*-acetylation. Among mature peripheral CD4 positive lymphocytes, the highly *O*-acetylated cells have low amounts of CD44 and high amounts of CD45RB indicating that *O*-acetylation may be higher among the naive cells. Whether this modification is specific to certain molecules and might play a role in CD4 lymphocyte biology is currently under investigation.

S35

Expression of Blood Group Glycolipids in Human Liver, Gall Bladder, Hepatic Artery and Portal Vein. Influence of the Secretor Gene on Antigen Expression

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Blood group ABO incompatible organ transplantation is justified in emergency situations (liver, heart) and in selected cases (kidney). Major ABO incompatible liver transplantation gives a long term graft function of about 25%. Increased knowledge of the expression of blood group carbohydrate antigens in human liver in relation to the donor's blood group ABO, Lewis and secretor phenotype/genotype is of importance to avoid antigen mismatch which will result in graft rejection.

Present study: Human livers obtained for transplantation but discarded for various reasons were collected. The vascular tree had been perfused with UW-solution removing plasma and blood cells, which is of importance to exclude contaminating glycolipid compounds in the liver tissue glycolipid fractions. Total neutral glycolipid fractions were prepared and analysed by TLC, immunostaining, and in selected cases by mass spectrometry and NMR spectroscopy.

Results: Blood group glycolipids with more than four sugar residues make up only a few percent of the total liver glycolipids. In contrast the gall bladder contains a large amount of complex blood group glycolipids. Immunostaining and structural data shows that the liver tissue and the biliary tissue of secretor individuals express only type 1 chain (Galβ1-4GlcNAc) based blood group antigens. This indicates that non-secretor individuals (20% of the population) will lack ABH antigens in their livers and this may explain the graft survival result of ABO incompatible transplantations. Analysis of tissues from a non-secretor individual is in progress.

S35

Oligosaccharide-Coated Liposomes: A Strategy of AIDS Vaccine with Emphasis on Cellular Immunity

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Recently, it has been suggested that cellular immunity plays an important role in protection against HIV. There is, however, no practical adjuvant which is safe for humans and is able to stimulate effective cellular immunity. We have prepared liposomes coated with neoglycolipids composed of lipids and various oligosaccharide chains. Ovalbumin (OVA) was enclosed in the liposomes as a model antigen and immunogenicity of these liposomes was examined by induction of a delayed-type hypersensitivity (DTH) response against OVA and production of anti-OVA antibody. Among neoglycolipids with various types of the oligosaccharide chains tested, only those with mannose residues at their non-reducing termini showed significant effects on the induction of cellular immunity. Since these neoglycolipids are composed of materials ubiquitously found in humans, oligomannose-coated liposomes are suggested to be a safe adjuvant for the induction of cellular immunity.

S35

Structures of Fucosylated Gangliosides with Linear Poly-*N*-Acetyllactosaminyl Chains Isolated from Normal Human Granulocytes

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Initial cell-cell adhesion of activated endothelial cells and granulocytes is mediated by selectins and their specific carbohydrate ligands. The endothelial leukocyte adhesion molecule (ELAM-1) binds to sialyl Le^x determinants. On neutrophils, this oligosaccharide is known to be present on N-glycans of glycoproteins as well as on glycosphingolipids.

One of the minor gangliosides was recently reported to carry the sialyl Le^x epitope (VI^3Neu5Ac,V^3Fuc-nLcOse$_6$Cer). As an extension of this work, we isolated a series of minor gangliosides from human granulocytes. Their structures were identified by methylation analysis (GC-MS), FAB-MS and MALDI-MS as well as by immunochemical overlay techniques with carbohydrate specific CSLEX1 and CDw65 antibodies. Ceramide deca-, undeca-, dodeca- and tridecasaccharides with three (nLcOse$_8$) and four (nLcOse$_{10}$) linear lactosaminyl repeats were identified. The ceramide portions were found to contain C_{18} sphingosine and predominantly $C_{16:0}$ fatty acids. All monosialogangliosides were homogeneous concerning their terminal α2-3Neu5Ac sialylation, but different in their fucosylation status.

Glycosphingolipid ligands for ELAM-1 are terminally sialylated lactosylceramides with a variable number of *N*-acetyllactosamine repeats and at least one fucosylated *N*-acetylglucosamine residue. Possible involvement of monosialogangliosides from neutrophils in the selectin-carbohydrate mediated process of cell-cell adhesion will be discussed.

S35

Rat Colonic Mucins Induce Autoantibody Against Colonic Mucin and Colitis-Like Damage in Mucosa

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Ulcerative colitis has been suggested to be one of the auto-immune diseases. However, auto-antigens have not been identified so far. We have reported that rat colonic mucins induce the anti colonic mucin antibody in rats, and that mucin immunized rats suffer from a colitis-like disorder about 8 weeks after first immunization. In the present study we perform a time course experiment to establish when colonic mucosal damage appears and investigate the location of the antigen using an immunohistochemical method.

Methods: Soluble mucins (S mucins) and membrane-bound mucins (M mucins) were purified as previously described from the supernatant and the membrane of Wistar rat colon tissue, respectively. Wistar male rats were sensitized by injection into the footpads with 200 μg S or M mucins emulsified with Freund's complete adjuvant. Three weeks later, they were boostered with 100 μg mucins emulsified with Freund's incomplete adjuvant. Pathological observation was performed using the 4, 6 and 8 week samples after first immunization.

Results: Colonic mucosal damage appeared 4 weeks after first immunization of both mucins in correlation with the appearance of an anti mucin antibody. The immunohistochemical study showed anti-S mucins sera detected surface mucosal mucins and anti-M mucins sera mainly detected goblet cell mucins.

Conclusion: Our results suggest that colonic mucins are an auto-antigen of ulcerative colitis.

S35

The Role of *O*-Glycosylated Receptors in Immune Response

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Participation of *O*-glycans on cells participating in immune response has been analysed using the lectin from *Amaranthus leucorapus* (ALL); this lectin interacts specifically with the T and Tn antigens. ALL possesses suppressive effect against particulated antigens, it induces inhibition of the phagocytic activity of murine macrophages and shows weak mitogenic activity against peripheral lymphocytes. Histochemical analyses indicated that ALL recognizes medullar thymocytes with the CD4+, CD3+ and CD45 Ra phenotype. Usually the GalNAcaSer/Thr structure is considered as typical for immature cells; however our results indicate that this structure seems to be present in unstimulated but mature cell populations, thus suggesting that the *O*-glycan core plays an important role in cellular interaction and differentiation.

This work has been supported in part by CONACyT and DGAPA-UNAM.

S35

Forssman Glycosphingolipid as an Immunohistochemical Marker for Murine Resident Macrophages Associated with Developing Haematopoietic Cells

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The immunohistochemical distribution of Forssman glycosphingolipid (Fo-GSL) in murine haematopoietic tissue was examined using a rabbit antiserum and a rat monoclonal antibody (Ab) which specifically reacted with Fo-GSL in liposome immune lysis assay. Bone marrow and splenic red pulp macrophages (Mϕs), which extend their cytoplasmic processes between developing haematopoietic cells, were stained with the Abs, whereas haematopoietic cells, alveolar Mϕs, Kupffer cells, and peritoneal Mϕs were not stained. Electron microscopy revealed that Fo-GSL was localized on the plasma membrane, mitochondial membrane, and cytoplasmic matrix. These Abs also stained central Mϕs of isolated eythroblastic islands, a rosette-like structure composed of a central Mϕ surrounded by erythroblasts. In bone marrow cultures, the Abs reacted with large flat F4/80$^+$ Mϕs attaching developing haematopoietic cells on their surface, but not F4/80$^+$ Mϕs derived from CFU-M and CFU-GM colonies. An allogenic bone marrow transplantation system enabled us to know that Fo-GSL is expressed on the resident Mϕs matured in the haematopoietic tissues. These results suggest that Fo-GSL$^+$ Mϕs are more functional in attaching developing haematopoietic cells than Fo-GSL$^-$ immature Mϕs.

S35

The Interaction of α_1-Acid Glycoprotein, its Carbohydrate Chains and Synthetic Glycoconjugates with Leukocytes from Human Peripheral Blood

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Immunomodulating activity of α_1-acid glycoprotein (AGP) is related to its glycosylation. The essential role of the carbohydrate chains in AGP activity was confirmed by the fact that AGP carbohydrate chains conjugated with polyacrylamide retained the immunomodulating activity of the natural glycoprotein [1].

To determine AGP target cells, we have studied the interaction of human peripheral blood leukocytes with AGP, its molecular forms differing in their glycosylation, AGP carbohydrate chains conjugates with polyacrylamide (OS-PAA) and their fragments (SiaLe^x-PAA, Le^x-PAA, etc.) labelled with fluorescein or without label. It was shown with the help of flow cytofluorometry that the main target cells for AGP itself were monocytes and granulocytes but not lymphocytes. Dose-dependent binding of Flu-labelled cells, inhibition of this binding by oligosaccharides of complex and hybrid types (tetra-, tri-, and biantennary N-chains) indicate that AGP binding with monocytes is mediated by a receptor which binds the branched sialooligosaccharides of complex type.

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S35

Sulfated Glucurono-Xylyloligosaccharides as Potent Heparin-Mimetic Inhibitors of the Cytotoxicity and Syncytium-Forming Infectivity of Human Immunodeficiency Virus (HIV-1) *In Vitro*

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The molecular mechanisms that underlie the pathophysiology of HIV-1 infection and the needed cure for AIDS are still elusive. Heparin-like molecules, likely players in these mechanisms, and various chemically sulfated polysaccharides inhibit HIV-1 *in vitro*, but their potential against AIDS in clinical applications was limited by toxicities of such non specific mixtures. We have shown [1, 2] that inhibition of the cytotoxicity (CT) and syncytium-forming infectivity (SF) of HIV-1 by a pharmaceutical mixture of sulfated xylans (SP54) was governed by structural specificity among the oligosaccharides (S-oligoS): minimum mass for high potency vs CT and SF (EC_{50} = ~300 ng ml^{-1}) was ≥5500, and one small oligoS (~nonoS) exhibited high activity vs CT only. Purified highly active structures were assayed *in vitro* vs HIV-1 and thrombin, analysed for GlcA content by reaction between *o*-nitrophenyl hydrazide and EDC-activated carboxyls, and analysed for molecular mass by sedimentation-equilibrium ultracentrifugation. S-oligoS had higher proportion of GlcA than the one GlcA per 10 xyloses in the original xylan and Courtauld models have suggested [1] that such GlcA residues might explain their heparin-mimetic nature. We now report a ~ tetradeca glycurono-xylyloligoS that contained up to three GlcA and exhibited the highest potency, EC_{50} vs CT = 200 ng ml^{-1} and EC_{50} vs SF = 50–100 ng ml^{-1}.

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2. Stone AL *et al.* (1992) *Glycobiology* **2**: 468.

S35

Effect of Ovine Submaxillary Mucin (OSM) on the Immune System in Mice

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The effect of OSM on the immune system was studied using C57BL/6 mice. OSM stimulated the production of antibody towards SRBC used as an antigen. The number of PFC in spleen of mice treated with low dose (2 μg per body) of OSM increased significantly, compared to untreated mice, but decreased at a high dose (200 μg per body). A similar effect was observed in the lymphocyte stimulation. OSM stimulated the proliferation of lymphocytes in spleen at a low dose, but suppressed them at a high dose. This lymphocyte stimulation due to OSM did not involve the increase in the population of T cells. The peritoneal macrophages were also stimulated by OSM, phagocytic activity of macrophages assayed using latex beads being augmented in OSM treated mice. The phagocytic activity of the macrophages in OSM treated mice was further reinforced by the addition of complements. The increase in the activity of macrophages caused by OSM seems to be correlated with the enhancement of IL-1 synthesis in macrophages, which could be demonstrated by determining IL-1 mRNA, amplified by RT-PCR. These results indicate that OSM is capable of activating the immune system of mice at low dose but the effect was reversed at high dose, and the activation deals with the macrophages rather than with T cells.

S35

Carbohydrate-Driven Mechanism in Non-Self Recognition by the Freshwater Prawn *Macrobrachium rosenbergii*

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Lectins in invertebrates have been involved in foreign-substance recognition. In crustaceans their participation in cellular cooperation or foreign particles-elimination has not yet been clearly established. In the freshwater prawn we have purified a lectin from serum which exerts specificity for 9,8,*O*-acetylsialic acid residues and glycoproteins. The lectin agglutinates only rat and rabbit erythrocytes and shows positive interaction with some bacteria containing acetylated sugars on their polysaccharide wall or capsule, such as *Aeromona, Bacillus cereus* and some *Pasteurella* strains; this lectin does not show opsonizating activity. Interestingly, granulocytic haemocytes show higher phagocytic activity against bacteria and erythrocytes with related specificity than the seric lectin. Our results suggest that the prawn possesses a seric and a cellular lectin and they could

participate in independent regulated mechanisms in the recognition and/or elimination of acetylated-sugar containing particles.

Supported in part by CONACyT, DGAPA-UNAM and UC-MEXUS programm (E.L.C).

S36. GLYCOSPHINGOLIPID INTERACTIONS AND RECEPTOR FUNCTION

Chairs: Ronald Schnaar, Cliff Lingwood

S36. 9.50am

Sialic Acid-Dependent Binding of *Helicobacter pylori* to Polyglycosylceramides of Human Erythrocyte Membranes

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It has been shown that some strains of *Helicobacter pylori* (human pathogenic bacterium implicated in gastrointestinal diseases) agglutinate human erythrocytes in a sialic acid-dependent manner [1]. The sialic acid-specific adhesin has been cloned and sequenced [2], but the erythrocyte receptor has not been identified, although several soluble sialyl conjugates are inhibitory. In our studies we tested different sialic acid-containing glycoconjugates from human erythrocyte membranes for their ability to react with the bacterium. The ganglioside fraction contained minor quantities of slow migrating components which bound *H. pylori* on silica gel TLC plates after overlay with ^{35}S-labelled bacterium. The main ganglioside species from human erythrocytes were, however, inactive when tested by the same method. Very strong reaction with *H. pylori* on TLC plates was observed for polyglycosylceramides (PGC). The PGC had typical branched polylactosamine structures and contained 1 sialic acid residue per about 14 glycosyl units [3]. The binding of *H. pylori* to PGC was abolished after treatment of PGC with mild acid, mild periodate or neuraminidase. Purified α3- and α6-sialylparaglobosides and brain gangliosides (GM_3, GM_1, GD_{1a}, GD_{1b}, GT_1, GQ_{1b} and GP_1) were inactive under the same experimental conditions. Glycoproteins from human erythrocyte membranes did not react with *H. pylori* when tested after electrophoresis by blotting and overlay with ^{35}S-labelled bacterium.

The results indicate that complex glycosphingolipids from human erythrocytes carry a sialic acid-containing epitope for *H. pylori* which is absent from gangliosides and glycoproteins.

1. Lelwala-Guruge J *et al*. (1992) *APMIS* **100**: 908.
2. Evans DG *et al*. (1993) *J Bacteriol* **175**: 674.
3. Miller-Podraza H *et al*. (1993) *Biochim Biophys Acta* **1168**: 330.

S36. 10.10am

Heat-Shock Proteins Bind Sulfogalactosylglycolipids: Role in Fertility and Bacterial Pathogenesis

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We have earlier identified sulfogalactolipid binding as an important component in sperm/egg recognition. A sulfoglycolipid binding protein (SLIP1) was isolated from spermatozoa and shown to be a potent inhibitor of fertilization *in vitro*. In addition we have shown that mycoplasmas which infect the reproductive tract and are associated with infertility similarly bind sulfogalactolipids *in vitro*. We have now demonstrated that a component within the SLIP1 preparation is the testis specific heat-shock protein. Similarly, recombinant mycoplasma HSP70 bovine brain (HSP 70) and HSP70 from bovine lung specifically bind sulfogalactosylceramide *in vitro*. Monoclonal antibodies against the HSP70 family specifically prevented the binding of intact mycoplasmas to sulfoglycolipids and EM studies of spermatozoa indicated the presence of cell surface HSP70s.

Helicobacter pylori is a gastric pathogenesis associated with ulcers. This organism binds to gangliotriaosyl and gangliotetraosyl ceramide and PE *in vitro* at neutral pH. However, when the organism is subjected to acid pH or brief heat-shock treatment the *in vitro* binding specificity changes to include sulfoglycolipids. This change in binding specificity is prevented in the presence of protein synthesis inhibitors. Binding to sulfotide but not PE following pH shock is inhibited in the presence of anti-HSP70 or anti-GRo-EL antibodies. Immuno EM shows the presence of *H. pylori* cell surface HSP expression following brief heat or pH shock. These studies indicate that heat shock proteins specifically recognize sulfoglycolipids in that these proteins can be expressed on the surface of both eukaryotic and prokaryotic cells to mediate the attachment to sulfoglycolipid bearing surfaces. We propose that *H. pylori* shows a two-stage receptor binding specificity in that initial binding within the stomach at acid pH involves cell surface heat-shock proteins binding to sulfoglycolipids immobilized within the protective mucous barrier. Subsequent penetration of this barrier to the neutral environment of the gastric mucosa facilitates a second receptor interaction with PE/Gg3/Gg4 to mediate attachment to host tissue.

S36. 10.30am

A Novel Protein Receptor on *Drosophila* Embryonic Glial Cells Binds Glycolipid Oligosaccharides

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Interactions between embryonic neural cells generate the specific patterns of connectivity observed in developed nervous systems. Cell surface carbohydrates have been proposed to function in the cell-cell recognition events necessary for the

formation of appropriate neural circuitry. One mechanism by which cell surface carbohydrates could mediate recognition requires that protein receptors exist on one cell that specifically bind carbohydrate moieties on an opposed cell's surface. We have undertaken an expression cloning strategy (based on [1]) to identify such endogenous lectins in *Drosophila* embryos. COS cells transfected with an embryonic *Drosophila* cDNA library were placed on surfaces adsorbed with glycolipids extracted from *Drosophila* embryos. Plasmids associated with adherent COS cells were harvested, amplified and rescreened. Through this 'panning' approach, a cDNA encoding a novel protein was obtained. The protein, designated NH3-2, mediates COS cell adhesion to *Drosophila* embryonic glycolipids and also induces heterophilic cellular aggregation when expressed in *Drosophila* S2 cells. By *in situ* hybridization and monoclonal antibody staining, NH3-2 expression is apparent in a subset of embryonic glial cells which are found at the midline of the developing ventral nerve cord and are crucial elements in guiding the formation of major axonal pathways. *In vitro* studies demonstrate that NH3-2 preferentially binds a subset of glycolipids purified from *Drosophila* embryos. The characterization of phenotypes associated with NH3-2 loss-of-function alleles will provide an *in vivo* assessment of carbohydrate and lectin function during neural development.

1. Aruffo A, Seed B (1987) *PNAS* **84**: 8573.

S36. 10.50am

Structure of the Complex of Verotoxin-1 and Globotriaosylceramide. Model Based on Binding Studies and Computational Docking

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Although the crystal structure of the B-subunits of VT-1 has been known for some time [1], the structure of the complex of the toxin and the receptor glycolipid, globotriaosylceramide (Galα1-4Galβ1-4Glcβ1-1Cer, Gb_3), has not yet been experimentally determined. In the present study molecular modelling based on the crystal structure of VT-1 and the favoured conformers of Gb_3 was used to predict the structure of the complex [2]. Our interest was focused on a region around residues 17, 18 and 30, which are involved in the binding site according to studies on specifically mutated verotoxins. Calculations with the GRID-program indicated that a pyranose ring could be accommodated in the crevice between Asp-17 and the hydrophobic exposed side chain of Phe-30. For the favoured conformer no.2 of Gb_3 the penultimate Galβ residue could be fitted into this crevice with its hydrophobic face in contact with the Phe side chain. In this orientation the Galα terminus of Gb_3 is in close contact with a loop containing aspartates 16-18. After energy minimization favourable hydrogen bonds were formed between hydroxyl groups of the terminal Galα1-4Galβ portion of Gb_3 and the residues Asp-17, Thr-21 and Lys-13 of the protein.

The structure of the complex proposed on the basis of the modelling is consistent with available binding data for mutated verotoxins. Furthermore, to probe the significance of specific hydroxyl groups in the Galα1-4Galβ portion of Gb_3, a series of deoxy analogues of Gb_3 has been synthesized. Results from VT1 binding studies with these deoxy analogues are in excellent agreement with the computationally predicted hydrogen bonding system between Gb_3 and the toxin.

The molecular modelling studies also suggest the presence of a second potential receptor binding site adjacent to Gly-62, Asn-32 and Phe-63 and the partially exposed surface of the side chain of Phe-30. Preliminary GRID-calculations indicate that a different conformer (no.6) of Gb_3 can be accommodated in this site. This second binding site may relate to the different Gb_3 binding characteristics of the other members of the verotoxin family.

1. Stein PE, Boodhoo A, Tyrrell GJ, Brunton JL, Read RJ (1992) *Nature* **355**: 748–50.
2. Nyholm PG, Brunton J, Lingwood CA (1995) *Int J Biolog Macromolecules*: in press.

S36. 11.10am

Apoptosis of Burkitt's Lymphoma Cells Induced Via Gb3/CD77, a Neutral Glycolipid Antigen:Intracellular Signalling Pathway

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Globotriaosylceramide (Gb3), a neutral glycosphingolipid, has a very restricted pattern of expression on haematopoietic cells since it is only expressed on a subset of tonsillar B lymphocytes and on Burkitt's lymphoma (BL) cells. It is now designated as CD77 and recognized as a B cell differentiation antigen. Several lines of evidence suggest that Gb3/CD77 plays a role in the apoptosis process of B cells, one of these points being that B subunits of verotoxin (VT-B), (the Gb3 specific binding subunits) are able to induce programmed cell death of CD77(+) BL cells. To further investigate the role of Gb3/CD77 and the mechanism of the signal transduced through this molecule, we have used VT-B and 38.13, an anti-CD77 mAb. We first show that when cross-linked by plastic-immobilized anti-IgM, 38.13 mAb is also able to induce apoptosis of CD77(+) BL cells: after 24 h the cells exhibited the morphological features associated with programmed cell death as well as the typical degradation of DNA to oligonucleosomal fragments. We also report that ligation of Gb3/CD77 induces an increase in the cytosolic $[Ca^{2+}]$, beginning after 2 min and sustained for at least 60 min. This rise is due to an influx of extra-cellular calcium and is a critical early step in the apoptosis process since its inhibition by EGTA protects the cells. Cross-linking of Gb3/CD77 also resulted in a regular increase of [cAMP]i until 30 min and the activation of a protein kinase A. These observations indicate that, although CD77 is not a transmembrane molecule, it is able to transduce a signal in BL cells.

S36. 11.30am

NANA-Lac Neoglycoproteins Inhibit EGF Receptor Autophosphorylation by Interaction with the External Domain of the Receptor

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Neoglycoproteins containing NANA-Lac (NeuAcα2 → 3Galβ1 → 4Glc) sugar chains were tested for their ability to inhibit autophosphorylation and tyrosine specific protein kinase activity of the epidermal growth factor receptor (EGF-R). NANA-Lac coupled to serum albumin was able to inhibit autophosphorylation of isolated EGF-Rs *in vitro*. Free NANA-Lac and Lac were not inhibitory. These data indicate that multivalency may be required for EGF-R inhibition. The serum albumin conjugates used here contained between 12 and 23 sugar chains per albumin molecule. Only about 10 μM NANA-Lac coupled to serum albumin was required for inhibition of EGF-R autophosphorylation. The NANA-Lac coupled complexes were also able to inhibit EGF receptor phosphorylation on intact cells. These data suggested that the NANA-Lac complexes interact with the external domain of the receptor since these complexes are not internalized by the cells. Consistent with this, NANA-Lac coupled to albumin was not able to inhibit recombinant EGF-R kinase domain (cytoplasmic) expressed in Sf9 insect cells. These data indicate that cell surface carbohydrate structures can influence the growth behaviour of cells through direct interaction with the external domain growth factor receptors.

S36. 11.50am

Modulation of a Myelin Protein Kinase by Multivalent Ganglioside Derivatives

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Myelin is a highly specialized membrane which forms an insulating barrier around axons. Previous studies have shown that gangliosides modulate protein kinase activities in isolated myelin membranes. However, these effects were seen only at high ganglioside concentrations, typically 50–150 μM. At these concentrations, it is difficult to determine whether these effects arise from a specific recognition event. We have synthesized multivalent ganglioside analogues by covalent linkage through the sphingosine amine to a protein carrier, bovine serum albumin. These *neoganglioproteins* offer two advantages over intact gangliosides: they are more hydrophilic, so they are less likely to have detergent-like effects, and multivalent presentation of the ganglioside may result in increased potency.

Rat brain myelin membranes were incubated in 50 mM Hepes pH 7.4, 10 mM $MgCl_2$, 300 μM $CaCl_2$, 200 μM EGTA, 0.03% Triton X-100 and 36.4 μM γ^{32}P-ATP at 30 °C for varying times with or without neoganglioprotein. Reactions were stopped with SDS and subjected to SDS-PAGE and autoradiography. Four bands, approximately 14, 20, 21, and 25 kDa were prominently labelled. Phosphorylation was linear for at least 15 min, and did not require calcium. Serine was the dominant phosphoamino acid. The phosphorylation of all four myelin protein bands was potently and differentially inhibited by the neoganglioprotein $GT1b_8BSA$, but not by GlcBSA, GalBSA, GalNAcBSA, ManBSA, or GT1b-oligosaccharide$_{12}$BSA. In contrast, neoganglioproteins did not modulate synaptosome-specific protein phosphorylation. The IC_{50} for GT1bBSA was 100–200 nM, at least 500-fold more potent than intact GT1b or GM1. The potency and specificity of these effects support the hypothesis that ganglioside recognition plays a signal transduction role in CNS myelin.

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S36 POSTERS

S36

Neutral Glycolipid Composition of Guinea Pig Large Vessels and Heart

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Guinea pig is a commonly used animal in transplantation research. Transplantation of a guinea pig heart to rat is the most common model for hyperacute xenograft rejection. Anti-donor antibodies with carbohydrate specificity have been suggested to be of importance for hyperactute rejection in pig to humans. We wanted to investigate the carbohydrate antigen composition of the donor animal in this xenotransplantation model. Aorta and large veins were chosen to provide a large amount of glycolipids representative of vascular tissue.

Heart, aorta, abdominal and caval veins were carefully dissected from 40 guinea pigs. Neutral glycolipids were prepared and analysed with thin layer chromatography, antibody reactivity, ^{1}H-NMR spectroscopy and mass spectrometry. The glycolipid composition of the great vessels was found to be very simple. Analysis of aortic neutral glycolipids showed presence of only gangliotriasylceramide and Forssman pentaglycosylceramide. In addition to this the vein preparation also contained cerebroside. Composition of guinea pig heart was more complex with expression also of glycolipids with chromatographic mobility in the two- and four sugar region.

The expression of large amounts of Forssman antigen in the large vessels as well as in the heart preparations is interesting as hyperacute rejection in a mouse to rat system is associated with the synthesis of an IgG anti-Forssman antibody [1]. Normal rat serum contains preformed IgM anti-Forssman antibodies [1]. These antibodies may be involved in the hyperacute rejection of a guinea pig heart, due to a reaction with Forssman antigen expressed on the luminal side of guinea pig endothelial cells.

1. Gustavsson ML *et al*. (1993) *Glycoconj J* **10**: 297.

S36

Ganglioside Effects on Signalling Mediated by Epidermal and Basic Fibroblast Growth Factor Receptor Tyrosine Kinases in Retinal Müller Glial Cells

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In order to analyse possible interactions between epidermal (EGF) and basic fibroblast growth factor (bFGF) receptors and membrane-bound gangliosides (CG), we examined the modulation of EGF and bFGF effects on retinal Müller glial cells (MG) following modification of their GC composition. Exogenous GG (GM3, GM1, asialoGM1 and GT1b) were incorporated into MG, and several aspects of cellular metabolism in response to EGF- and bFGF-receptor activation were studied: membrane fluidity, global phosphotyrosine levels, growth factor binding and tyrosine phosphorylation of EGF- and bFGF-receptors and their cellular substrates. All of these parameters (except binding) were influenced to different extents by GG pre-treatment: particularly, the kinetics of receptor tyrosine phosphorylation were modified by changes in GM3, the major endogenous GG of MG. AsialoGM1 also inhibited EGF- and bFGF-receptor tyrosine kinase activity, whereas the other GG tested had less or no effect. We also observed that GM3 pre-treatment of MG inhibited EGF- and bFGF-induced activation of MAP kinase, as assayed by myelin basic protein phosphorylation. These data show that GG modulate short-term effects of neutrophic factors by altering receptor tyrosine kinase activity.

S36

Signal Responsibility of Monosialoganglioside (GM3) Reconstituted in Sphingomyelin and Glucosylceramide Membranes

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GM3 usually exists surrounded with a different kind of lipid such as a phosphatidylcholine or a neutral glycolipid in plasma membrane. The effect of such a matrix lipid on the recognition of GM3, however, has not been investigated so far. In this study, we investigate recognizabilities of GM3 in the mixed monolayer with GlcCer and SM against sugar-binding proteins such as wheat germ agglutinin (WGA). Bindings of WGA to GM3-containing membranes were quantitatively monitored by a Quartz crystal microbalance. Influences of matrix lipids on the recognizability of GM3 were discussed from maximum binding amount (Δm) and initial bonding rate (V) of WGA.

At low content (less than 10 mol%) of GM3 in the matrix, the Δm and V are largely different between GlcCer and SM matrix membranes. Incorporation of GM3 in GlcCer membrane resulted in a drastic increase in lectin binding in comparison with the GM3/SM membrane. Such a high recognizability of GM3 in the GlcCer matrix membrane at low GM3 content must be important for the expression of antigenicity of ganglioside on plasma membrane, because the content of ganglioside in plasma membrane is usually a small percentage. The present study assisted us to understand that phosphatidylcholine makes the recognition of GM3 cryptic, and glucosylceramide is the essential lipid for high recognition of GM3.