Glycoconjugate Journal (1996) **13:** 879–904

Abstracts of the 4th Jenner International Glycoimmunology Meeting

HOTEL POSEIDON CLUB, LOUTRAKI, GREECE, 12–15 NOVEMBER 1996

SESSION 1: GLYCOBIOLOGY – THE BASICS
CHAIRPERSONS: JOHN AXFORD & JAMES PAULSON

S1.1

Novel pathways in complex-type oligosaccharide synthesis. New vistas opened by studies in invertebrates.

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Protein- and lipid-linked complex-type oligosaccharide chains typically contain Galβ1→4GlcNAc (LacNAc) units as building blocks of their so called backbone regions. These backbones are the carriers of the terminal structures that confer specific properties on the glycoconjugates carrying them. In an increasing number of cases, however, a GalNAcβ1→4GlcNAc (*N,N'*-diacetyllactosediamine, LacdiNAc) unit is found in stead of the LacNAc unit in these backbones. We have characterized novel UDP-GalNAc:GlcNAcβ-R β1→4-*N*-acetylgalactosaminyltransferases (β4-GalNAcT) in the schistosome *Trichobilharzia ocellata* and the snail *Lymnaea stagnalis* that appear to be involved in the biosynthesis of such LacdiNAc-type oligosaccharide chains. These β4-GalNAcTs control the LacdiNAc pathway of complex-type oligosaccharides in these organisms. They act analogously to mammalian β4-galactosyltransferase (β4-GalT) and share with this enzyme its acceptor properties and, sometimes, the responsiveness to α-lactalbumin. In view of these results we anticipated that β4-GalT and β4-GalNAcT would show molecular similarity that could be exploited to isolate DNA sequences coding for β4-GalNAcT by cross hybridization using β4-GalT cDNA as a probe. Indeed a cDNA could be isolated from a snail library coding for a protein with a domain structure typical for a glycosyltransferase (GT) and showing considerable similarity with β4-GalT. Unexpectedly, expression of this cDNA resulted in the production of an enzyme that catalyzes the transfer of GlcNAc, rather than Gal or GalNAc, in β1→4-linkage to β-*N*-acetylglucosaminides. This novel β4-*N*-acetylglucosaminyltransferase (β4-GlcNAcT) has not been described before and its existence could not be predicted from known oligosaccharide structural data. The organization of the β4-GlcNAcT gene shows remarkable similarities to that of β4-GalT. Interestingly, two exons show a high degree of similarity to part of the preceeding exon suggesting that they have originated by exon duplication. The corresponding 'single-copy' exon in β4-GalT has been implicated in nucleotide-sugar binding. Deletion of the two repeated exons in the snail gene resulted in a β4-GlcNAcT with slightly altered sugar-donor specificity. The full length β4-GlcNAcT shows essentially no chitin synthase activity, but rather acts on β6-linked GlcNAc residues on O-linked core 2 and 4 glycans and branching points of blood-group I-active substances. Therefore, it may function in another variant of complex-type oligosaccharide synthesis, the 'Chitobio pathway', which might be common in invertebrates. The extensive sequence similarity found for β4-GalT and β4-GlcNAcT cDNAs and the properties shared by β4-GalT and β4-GalNAcT suggest that these three enzymes might be evolutionary related and represent members of a novel GT family. Interestingly, several species have appeared to contain two or more different transcripts that may encode candidate members of this family.

S1.2

Murine β1,4-Galactosyltransferase: Recruitment of a Housekeeping Gene from the Vertebrate Gene Pool for a Mammary Gland-Specific Function.

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β1,4-galactosyltransferase (β4-GT) is a constituitively expressed trans-Golgi resident, type II membrane-bound enzyme, that catalyzes the formation of the β4-N-acetyllactosamine (Galβ4-GlcNAc) and poly-N-acetyllactosamine structures in glycoconjugates. β4-GT enzymatic activity is widely distributed in the vertebrate kingdom and has also been demonstrated in members of the plant kingdom. The plant and animal kingdoms are estimated to have diverged about one billion years ago. In invertebrates, genomic fragments with significant sequence in common with β4-GT from vertebrates have been reported for a number of evolutionarily distant organisms including C. elegans.

In mammals, β4-GT has been recruited for a second biosynthetic process, the synthesis of lactose (Galβ4-Glc), which takes place exclusively in epithelial cells of the mammary gland of lactating animals. This change in acceptor sugar specificity for β4-GT is effected by interaction with the non-catalytic milk protein, -lactalbumin. In preparation for lactose synthesis, beginning in mid- to late pregnancy, -lactalbumin is expressed *de novo*, while β4-GT enzymatic levels are increased at least 10-fold over constituitive levels. Support for the notion that during the evolution of mammals, the β4-GT gene was recruited directly from the existing pool of functional vertebrate genes, is provided from the observation that β4-GT from non-mammals, including the chicken, can interact with -lactalbumin to synthesize lactose in vitro.

We have shown in mammals that transcription of the murine β4-GT gene in somatic tissues occurs from two start sites separated by ~200 bp. Use of these two transcriptional start sites results in mRNAs of 3.9 and 4.1 kb. The main difference between these two mRNAs is the length and extent of the predicted secondary structure of the respective 5'-untranslated regions. In somatic tissues, including the virgin mammary gland, the 4.1 kb start site is predominantly used. The only exception to this general pattern is found in the mammary gland from mid- to late pregnant and lactating animals, where the 3.9 kb start site is preferentially used. In contrast, transcription of the β4-GT gene from a non-mammalian vertebrate, the chicken, takes place at a

single start site. These observations, combined with a promoter deletion analysis, using β4-GT/CAT hybrid constructs, footprinting and gel shift studies experimentally support a model of transcriptional regulation in which the distal region upstream of the 4.1 kb start site functions as a housekeeping promoter, while the proximal region upstream of the 3.9 kb start site functions primarily as a mammary cell-specific promoter. The essential feature of our model is that mammals have evolved a two-step mechanism to generate the elevated levels of β4-GT enzymatic activity, in the lactating mammary gland, that are required for lactose biosynthesis. In step one, there is an upregulation of the steady state levels β4-GT mRNA, due to increased transcription from the 3.9 kb start site. In step two, the 3.9 kb β4-GT transcript is translated more efficiently, relative to its housekeeping counterpart, due to deletion of most of the long GC-rich 5′-untranslated sequence characteristic of the 4.1 kb mRNA. Collectively, these observations support the conclusion that the 3.9 kb start site along with its accompanying tissue-restricted regulatory elements were introduced into the vertebrate β4-GT gene, during evolution of mammals, as a direct consequence of the recruitment of this galactosyltransferase for the mammary gland-specific biosynthesis of lactose.

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S1.3

Defective glycosyltransferases are not good for your health

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Five genetic diseases are associated with defective Asn-linked glycan synthesis: Inclusion Cell Disease, Leukocyte Adhesion Deficiency Type II, Congenital Dyserythropoiesis Type II (HEMPAS) and Carbohydrate-Deficient Glycoprotein Syndromes (CDGS) Types I and II. CDGS are multisystemic diseases with malformation of the nervous system. CDGS I is due to loss of phosphomannomutase activity [1] with decreased mannose incorporation into dolichol pyrophosphate oligosaccharide. CDGS II is an autosomal recessive disease due to point mutations in the catalytic domain of UDP-GlcNAc:α-6-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase II (GnT II) [2, 3]. The GnT II gene (*MGAT2*) is localized to chromosome 14q21. Restriction endonuclease analysis of 23 blood relatives of a CDGS II patient revealed 13 heterozygotes with reductions (33 to 68%) in mononuclear cell GnT II activity. Embryonic lethality with defective neural tube formation occurs at day 10.5 in null mice lacking a functional GnT I gene [4, 5]. Data from humans and mice thus indicate that complex N-glycans are essential for normal neurological development. HEMPAS is a multifactorial disease; at least one case has been attributed to GnT II deficiency [6]. HEMPAS erythrocyte band 3 shows complete absence of poly-*N*-acetyllactosamine (PL) whereas CDGS II band 3 PL is reduced 50% [3]. Unlike CDGS II, HEMPAS is a relatively mild disease with no apparent neurological involvement. CDGS II erythrocytes do not show the serology typical of HEMPAS. The diseases are clearly different. 1. Van Schaftingen E, Jaeken J (1995) *FEBS Lett* **377**: 318-320. 2. Jaeken J, Schachter H, Carchon H, Decock P, Coddeville B, Spik G (1994) *Arch Dis Child* **71**: 123-127. 3. Charuk JHM, Tan J, Bernardini M, Haddad S, Reithmeier RAF, Jaeken J, Schachter H (1995) *Eur J Biochem* **230**: 797-805. 4. Ioffe E, Stanley P (1994) *Proc Natl Acad Sci USA* **91**: 728-732. 5. Metzler M, Gertz A, Sarkar M, Schachter H, Schrader JW, Marth JD (1994) *EMBO J* **13**: 2056-2065. 6. Fukuda MN (1990) *Glycobiology* **1**: 9-15.

S1.4

New Approaches for Probing Carbohydrate-Protein Interactions

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It is now well documented that many organisms depend upon cell-surface carbohydrate moieties for normal function. In many cases the oligosaccharide appears to perform a recognition function, the molecular details of which have remained obscure in all but a few systems. A prerequisite to understanding these phenomena, and in particular the design of novel chemotherapetic agents which inhibit the recognition function, is knowledge of the structure, conformation and dynamics of the free ligand. Ideally, the bound-state conformation of the ligand and the architecture of the binding site is also required for a rational design of novel inhibitors. Recent techniques utilising high-resolution multidimensional, multinuclear NMR will be described, whose aim is to define the solution dynamics of the free ligand and also the bound state conformation in complex with various protein receptors. These techniques will be illustrated for a variety of systems including a glycoconjugate bound to an antibody Fv fragment and the glycolipid receptors of the AB_5 class bacterial toxins.

S1.5

Carbohydrate Moiety of *Plasmodium falciparum* Glycoproteins.

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Metabolic labelling of *Plasmodium falciparum* parasites with [^{3}H]GlcN, [^{3}H]Gal and [^{3}H]ethanolamine, and subsequent purification by SDS-PAGE of the labelled material provided effective labelling of the MSP-1, 195 kDa, and MSP-2, 42-53 kDa, glycoproteins. Reductive β-elimination of the MSP-2 released from the gel consisted of glycopeptides containing labelled surgars. Identification of the sugar residues demonstrated the presence of N-acetylglucosaminitol and trace of N-acetylgalactosaminitol amongst other labelled sugars. Reductive β-elimination with sodium hydroxide-sodium borotritide-borohydride showed the presence of N-actyglucosaminitol and alanine in the hydrolysis products. The MSP-2 was retained on solid phase wheat-germ agglutinin and was released from the lectin by treatment with GlcNAc. Upon treatment with exoglycosidases the MSP-2 and derived oligosaccharides released labelled components. Labelled Gal was incorporated into the MSP-2 using [^{3}H]UDP-Gal and lactosyltransferase. The galactosylated glycoprotein released labelled Gal upon treatment with β-galactosidase. Dephosphorylation of the MSP-2 with mild HF treatment released the core glycans. Treatment of the core glycans with α-galactosidase released labelled sugar corresponding to standard galctose. Incorporation of sugars and growth of *P. falciparum* were restricted by mannosamine. These results suggest that the carbohydrate chains of the MSP-2 are attached to the protein backbone via GlcNAc-serine/threonine in O-glycosyl linkage and the glycoprotein has mainly of short chains linked to the protein core.

S1.6

Post-translational Generation of a Soluble Form of the Macrophage Mannose Receptor: Dependence on IL-4 and Phagocytosis. L. MARTINEZ-POMARES, J.A. MAHONEY, and S. GORDON. *Sir William Dunn School of Pathology, University of Oxford, Oxford UK.*

The macrophage mannose receptor (MR) is a 175 kDa type I integral membrane glycoprotein, specifically expressed on the cell surface of macrophages (MØ), dendritic cells, and hepatic endothelial cells. MR expression on MØ is greatly increased after treatment with the Th2 cytokine IL-4. Known physiological roles of MR include endocytic clearance of mannose-terminated glycoproteins, and phagocytosis of a variety of unopsonized micro-organisms. We have discovered a novel soluble form of the receptor, which appears to be generated by a specific post-translational cleavage event. Western blot analysis of conditioned media from murine Biogel-elicited peritoneal MØ using a polyclonal anti-MR antibody demonstrated the presence of a smaller, soluble form of MR (sMR). Pulse-chase and immunoprecipitation experiments showed that sMR is generated post-translationally; it was detected after 2 hr of chase, and continued to accumulate up to 18 hr. Experiments with carbohydrate-derivatized beads demonstrated that MR carbohydrate binding activity and specificity are maintained in sMR. Synthesis of both intact MR (as previously reported) and sMR was greatly enhanced by IL-4 treatment. Interestingly, when zymosan, a yeast cell wall particle known to stimulate macrophage phagocytosis, was added to IL-4-treated cultures during the chase period, production of sMR was enhanced. These data suggest that generation of sMR, presumably via a proteolytic cleavage event, is specifically upregulated during phagocytosis. Supported by the British Heart Foundation.

S1.7

GLYCOSYLTRANSFERASE EXPRESSION DURING THE MATURATION OF NORMAL HUMAN MYELOID CELLS

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Cell surface carbohydrates are important in the adhesive interactions of haematopoietic cells. The Lewis^X antigen or CD15 was the first carbohydrate structure to generate interest in neutrophil glycosylation (1). More recently the sialyl Lewis^X antigen which is present on the surface of myeloid cells and is a ligand for E-selectin has been shown to mediate neutrophil binding to inflamed endothelia (2). The cell surface expression of sialylLewis^X and Lewis^X antigens changes during myeloid cell maturation (3) and is likely to be regulated by competition between α1-3 fucosyltransferases (FT) and βgalα2,3 or α2,6-sialyltransferases (ST3 and ST6) which utilise the same Galβ1-4GlcNAc-R precursor structure. It has been suggested that ST6 plays a role since its marked reduction in activity following DMSO-induced maturation of HL60 is accompanied by the appearance of sialylLewis^X on the cell surface (4). To define the changes that occur during the development of normal cells we studied cell fractions of normal bone marrow: CD34+ progenitor cells, promyelocytes, metamyelocytes and, from peripheral blood, neutrophils. In each fraction we measured levels of FT, ST6 and ST3 and we quantified the level of expression of mRNA of FTIV, FTVII, ST6 and ST3 by quantitative RT-PCR. We observed an increase in FT activity in promyelocytes but ST activity remained stable in BM fractions. All activities dropped in neutrophils. The level of expression of mRNA for FTIV, FTVII, ST3 and ST6 remained stable throughout maturation with a decrease of ST6 and a significant drop of FTIV in neutrophils. We did not find a direct correlation between mRNA level and activity of the corresponding enzyme.

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2- Lowe *et al.*, Cell 1990 **63:**475
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S1.8

Conversion of the carbohydrate moiety of fungal glycoproteins to a mammalian type

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To make production of therapeutically important glycoproteins in fungi more attractive, ways to change fungi-like glycosylation to the mammalian type were investigated. Acceptor substrate for recombinant human N-acetylglucosaminyl transferase I was found on extracellular proteins from the filamentous fungi *Trichoderma reesei* and *Aspergillus niger*. Further "in vitro" incorporation of galactose and sialic acid by the corresponding mammalian glycosyltransferases was also possible. N-glycans of *A. niger* α amylase and *T. reesei* cellobiohydrolase I were enzymatically released and structurally analyzed in order to confirm the GlcNAc accepting compound to be $Man_5GlcNAc_2$ [1]. Primary structures of the more abundant N-glycans were also elucidated after fractionation according to size by Biogel P4 gelfiltration. Different techniques were used, namely : PAGE after ANTS labelling of the oligosaccharides [2] , HPAEC-PAD [3], ^1H, ^{13}C and ^{31}P NMR analyses [4]. Following N-glycans were recognized on *T. reesei* CBH I : $GlcMan_8GlcNAc_2$, $GlcMan_7GlcNAc_2$, $Man_7GlcNAc_2$, $ManPMan_7GlcNAc_2$, $GlcMan_5GlcNAc_2$ and $Man_5GlcNAc_2$. On *A. niger* α amylase, the predominant oligosaccharide was recognized to be $Man_5GlcNAc_2$. From these results, it was suggested that in *T. reesei,* trimming by glucosidase II was not efficient. On the other hand, the presence of a fully active α-1,2-mannosidase was suggested . Growth conditions and/or the strain used (*T. reesei* RUTC 30) were thought to be responsible for the observed trimming patterns. The knowledge of above mentioned experiments is used during the continuation of this research : the aim is to manipulate filamentous fungi, so that more acceptor substrate for mammalian GlcNAc-transferase I is synthesized "in vivo".

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S1.9

Physical parameters for the native, intermediate and denatured states of glycosylated and aglycosylated mouse IgG2b.

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Glycosylated and aglycosylated Fc fragments of mouse IgG2b have been investigated using scanning microcalorimetry, circular dichroism and hydrodynamic methods. At pH 4.8 and 20° the fragments have slightly differing partial specific heat capacity values but insignificantly differing CD spectra in the near UV. At lower pH values or higher temperatures transition to a denatured state proceeds via an intermediate state in which the co-operative structure of the C_H2 domain disintegrates whilst that of the C_H3 domain is retained. An intermediate state result at pH 2.8, 20° in which the C_H2 domains of both the glycosylated and aglycosylated have melted.

A different intermediate state arises at pH 4.8 and 53° in which the C_H2 domains melt and the C_H3 domains retain their co-operative structure and far UV CD spectra indicates that the glycosylated Fc fragments retains more secondary structure than the aglycosylated form. The near UV spectra indicate a more asymmetric environment for tryptophane and tyrosine residues in the glycosylated fragment, relative to the aglycosylated protein, suggestive of a more compact structure.

These data suggest that glycosylation of the C_H2 domain results in generation of a more compact structure than for the non-glycosylated protein, as evidenced by the fact that each enters the molten globule state under differing conditions

881S1.10

O-Glycosylated IgG in Rabbit Serum Decreases with Hyperimmunization. L.M. VASCONCELOS*, A. PANUNTO-CASTELO, L.M. GUIMARÃES-SANTOS & M.C. ROQUE-BARREIRA. *Dep. Pathology, CCBi, UFAL, Maceió. *Dep. Immunology, Faculty of Medicine of Ribeirão Preto, Brazil.*

The most outstanding property of jacalin, a specific lectin for Galß1-3GalNac is interaction with human IgA_1. We reported that jacalin selectively binds to rabbit IgG; 30% of normal rabbit IgG prepared by protein A affinity is adsorbed to jacalin, an interaction attributed to the presence of O-glycosylation in the hinge region of a rabbit IgG subpopulation. Chromatography of other IgG samples from different rabbits showed variability in the proportion of IgG adsorbed to Sepharose-jacalin, which was not correlated with the specificity of the immunoglobulins assayed. We then evaluated the association of jacalin binding with the extent of immunization of the rabbits from which the antibodies were obtained, using anti-goat IgG from rabbit sera arbitrarily classified as (1) immune (collected one month after the beginning of the immunizing protocol) or (2) hyperimmune (collected six months after the beginning of the protocol, with periodic boosters). Normal sera (3) were collected from non-immunized health rabbits. The gamma-globulin fraction (50 mg) of each rabbit serum was applied to a Sepharose-jacalin column (10 ml). Material from normal sera reproduced the initial observation of 30% binding to immobilized jacalin. With the gamma-globulin from immune sera, 17 to 20% of the protein was retarded, whereas with the gamma-globulin from hyperimmune sera only 6 to 10% of the protein was adsorbed to jacalin. The variability (6 to 30%) observed in the proportion of IgG that binds to jacalin in the sera studied is similar to the variability in the proportion of O-glycosylated IgG molecules (2 to 34%) in New Zealand rabbits. This coincidence in variability suggests the occurrence of overlapping between the rabbit IgG populations that bind to jacalin and the O-glycosylated rabbit IgG. Thus, the proportion of O-glycosylated IgG, that binds to jacalin, is inversely correlated with the extent of immunization of the animals from which the antibodies are obtained.

S1.11

SO_3-3Galβ1-3GlcNAc distribution in the human body.

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Using a panel of synthetic oligosaccharides attached to a polyacrylamide carrier, the epitope of monoclonal antibody F2, evoked to high M_r salivary mucins, was mapped to the SO_3-3Galβ1-3GlcNAc- moiety of the sulfo-Le^a antigen. Using immunochemical techniques, the expression of the F2-epitope was investigated in a number of different isolated human mucin species, as well as in human and rat tissue specimens. The mAb F2 bound to high M_r salivary mucins, cervical mucins, colon mucins and gallbladder mucins, but not to low M_r salivary mucins nor to gastric mucins. Immunohistochemical screening of human tissues with mAb F2 revealed a positive reaction with a number of epithelia, including the (sero)mucous salivary glands, the goblet cells of the colon, the lining epithelium of cervical and esophageal glands, the suprabasal skin keratinocytes, and Hassall's corpuscles of the thymus. No staining was found in normal breast, pancreas, small intestine, spleen, and lymph nodes. Normal gastric glands were negative, but gastric intestinal metaplastic glands strongly stained with the antibody. In rat tissues, mAb F2 labeled epithelial cells of salivary glands, colon and stomach. In addition to epithelial cells, extracellular matrix components in rat thymus and skin were labeled by mAb F2. No labeling of erythrocytes, granulocytes, lymphocytes or bone marrow cells was found by FACScan analysis. The present data shows a tissue specific distribution of the F2-epitope in cells from the epithelial lineage in human and rat.

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S1.12

Endogenous fucosyltransferase activities in COS cells

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In the belief that COS cells are free from endogenous fucosyltransferase activity, or cell surface expression of fucosylated antigenic structures, these cells have frequently been used for transient expression of fucosyltransferase genes believed to be involved in the biosynthesis of ligands for the Selectins. However, in transfection experiments in which we employed stringent detection conditions because of very low levels of expression of the transfected cDNA, we observed weak sialyl-Le^x antigenic expression on the cell surface and low levels of fucosyltransferase activity in cell extracts of the control untransfected COS-7 cells [1]. Similar activities were detected in three specimens of COS-7 cells and two specimens of COS-1 cells. Since activation of endogenous α1,3-fucosyltransferase genes in CHO cells has been shown to complicate the characterisation of transfected fucosyltransferase genes [2], we decided to examine the endogenous activities in COS cells in greater detail. Substrate specificity tests revealed activity with *N*-acetyllactosamine and asialo-fetuin, but little activity with sialylated acceptors. However, whereas the activity with *N*-acetyllactosamine had a pH optimum of 7-7.5, the optimum with asialo-fetuin was pH 5.5, suggesting the presence of two different fucosyltransferases. Both activities were relatively resistant to inactivation with NEM, but the enzyme transferring fucose to asialo-fetuin was considerably more sensitive to heat inactivation than the one utilising *N*-acetyllactosamine. Northern blot analysis of mRNA from COS-7 cells probed with [^{32}P]labelled cDNAs corresponding to the five cloned fucosyltransferase genes, Fuc-TIII, Fuc-TIV, Fuc-TV, Fuc-TVI and Fuc-TVII, failed to demonstrate any transcripts and a dot blot analysis of concentrated mRNA with these same probes gave a weak indication of homology with only Fuc-TVII.

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S1.13

Eliminating Matrix Interference in Monosaccharide Analysis

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The monosaccharide content of a glycoprotein is often determined by acid hydrolysis at elevated temperature. For glycoproteins with low levels of glycosylation, monosaccharide quantitation can be compromised from fouling of the working electrode surface by amino acids. Lysine elutes on the CarboPac PA1 column just prior to galactosamine whereas the remaining amino acids and most peptides elute after the monosaccharides and do not interfere with monosaccharide quantitation (the exception being arginine which elutes near the column void and does not affect monosaccharide detection). A direct comparison of PAD (using the standard carbohydrate waveform) vs. Abs_{215} detection of lysine reveals that lysine does not cleanly come off the working electrode. The inhibition of response of monosaccharides caused by lysine could be corrected for by the post hydrolysis addition of a rhamnose internal standard[1] and the determination of "correction factors". To eliminate the need for correction factors, we have developed a guard column with an altered selectivity for amino acids which, when used with a new separator, causes lysine to elute after the monosaccharides and also causes hydrophobic amino acids to elute further after the monosaccharides. The new separator and guard columns solve the lysine fouling problem, reduce baseline noise, and eliminate the need for correction factors. The new separator no longer has an O_2 dip which can interfere with low or sub-pmol detection of monosaccharides. By manipulating resin and latex chemistry, the O_2 dip has been moved well after the monosaccharides. The new guard column and new separator significantly improve the accuracy, ruggedness, and sensitivity of glycoprotein monosaccharide analysis using HPAE-PAD.

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S1.14

MODIFICATION OF THE UDP-SUGAR SPECIFICITY OF THE *LYMNAEA STAGNALIS* β1→4-N-ACETYL-GLUCOSAMINYLTRANSFERASE

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The *Lymnaea stagnalis* β1→4-N-acetylglucosaminyltransferase (β4-GlcNAcT) is an enzyme with structural similarity to mammalian β1→4galactosyltransferases (β4-GalT) (1). Also the exon partition of the genes encoding these enzymes is very similar. The main difference is that the β4-GlcNAcT gene contains three exons, encompassing additional sequences that are absent in the β4-GalT gene. Two of these exons (7 and 8) show a high sequence similarity to part of the preceding exon (exon 6), suggesting that they have originated by exon duplication. The exon in the β4-GalT gene, corresponding to β4-GlcNAcT exon 6, encodes a region that has been proposed to facilitate the binding of UDP-Gal. The question therefore arose, if the repeating sequences encoded by exon 7 and 8 of the β4-GlcNAcT gene would determine the specificity of the enzyme for UDP-GlcNAc. It is shown that deletion of only the sequence encoded by exon 8 resulted in a completely inactive enzyme. On the other hand, deletion of the amino acid residues encoded by exon 7 and 8 resulted in an enzyme with an elevated kinetic efficiency for both UDP-GlcNAc and UDP-GalNAc. The mutant enzyme was shown to catalyse the transfer of GlcNAc and GalNAc with an equal maximal velocity. These results suggest that the insertion in the β4-GlcNAcT, and its surroundings, are involved in interaction of the enzyme with its sugar-donor.

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S1.15

Structural features of human salivary mucin (MUC7). T. L. GURURAJA, N. RAMASUBBU, P. VENUGOPALAN, M. S. REDDY, K. RAMALINGAM and M. J. LEVINE *(Department of Oral Biology, SUNY at Buffalo, Buffalo, NY, USA)*

Human salivary mucin (MUC7) is characterized by a single polypeptide chain of 357 amino acids including the presence of six tandem repeats each consisting of 23 amino acids. A unique feature of the tandem repeats is the high (33%) proline content. Specifically, the tripeptide sequence, Ala-Pro-Pro occurs 18 times within the tandem repeats. To understand the role of this tripeptide sequence on MUC7 conformation, Boc-Ala-Pro-Pro-OBzl (APP) was synthesized by standard solution phase methods and its conformation was determined using X-ray diffraction. The APP segment exhibit a poly-L-proline type II conformation. The poly-proline structure adopted by the APP segments, dispersed throughout the MUC7 backbone, may impart a stiffening of the backbone and could act in consort with the glycosylated segments to keep the mucin molecule in a semi-rigid rod shaped conformation. CD analysis of the secondary structure of MUC7 and several APP-poor tryptic peptides provided additional evidence that the APP segments in the tandem repeats play a significant role in the conformational dynamics of MUC7. Definitive chemical analysis indicated that 83% of Ser/Thr residues are O-glycosylated. The semi-empirical prediction algorithm for O-glycosylation indicated that Ser/Thr in the tandem repeats displayed >0.75 probability for O-glycosylation. The aforementioned data and additional analysis of the MUC7 primary structure indicated that this mucin is divided into five distinct domains/regions: 1) an N-terminal basic, histatin-like domain which has a leucine-zipper segment; 2) a sparsely glycosylated domain; 3) six heavily glycosylated tandem repeat domain containing APP segments; 4) another heavily glycosylated MUC1,2-like domain without APP segments; and 5) a C-terminal leucine-zipper segment. A new structural model for MUC7 will be presented. Supported by USPHS Grants DE07585 and DE08240.

SESSION 2: OLIGOSACCHARIDES AND PROTEIN RECOGNITION (PART I)
CHAIRPERSONS: ELIZABETH HOUNSELL & OLE HINDSGAUL

S2.1
The Structure of a Human Rheumatoid Factor Bound to IgG Fc

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Rheumatoid factors (RF) are autoantibodies found in the sera and synovia of patients with rheumatoid arthritis (RA). RFs most commonly belong to either the IgM or IgG class, and recognise epitopes in the Fc regions of IgG, thus forming immune complexes. We have employed X-ray crystallography to study in detail the nature of this autoantibody-autoantigen interaction, and have determined the crystal structure of a complex between the Fab fragment of a human IgM RF (RF-AN) derived from a patient with RA, and the Fc region of human IgG4. The complex has a stoichiometry of 2:1 Fab:Fc, and each RF is found to bind to an epitope that consists of residues from both the Cγ2 and Cγ3 domains. The topology of the interaction with respect to the antibody however, is unlike that of any other complex between an antibody and a protein antigen seen to date, for only one edge of the potential combining site surface is involved. The number of contact residues is also small, consistent with the low affinity. Only two contact residues are contributed by the (λ) light chain, but one of these, which makes critical contacts with the antigen, is somatically mutated from the germline gene providing evidence for a process of antigen-driven selection in the generation of this RF. The epitope recognised by RF-AN is very similar to the binding sites in IgG Fc for the bacterial proteins A and G, and the disposition of these epitopes will be discussed in relation to functional properties of the IgM including complement activation by immune complexes, and the activation of B cells via membrane-bound IgM.

S2.2
SIALIC ACIDS AS LIGANDS IN RECOGNITION PHENOMENA.

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The sialic acids are monosaccharides typically found at the outermost ends of the sugar chains of glycosylated molecules (glycoconjugates). Combinations of various substitutions and linkages to the underlying sugar chain generate a remarkable diversity in the structural variations and presentations of sialic acids. This diversity appears to be expressed in a molecule-specific, tissue-specific and developmentally-regulated fashion. Sialic acids can inhibit intermolecular and intercellular interactions by virtue of their negative charge and terminal location. However, they can also act as critical components of ligands that are recognized by a wide variety of proteins of animal, plant and microbial origin. Recognition can be affected by specific structural variations and modifications of sialic acids, their linkage to the underlying sugar chain, the structure of these chains, and the nature of the glycoconjugate to which they are attached. This talk will provide a brief summary of the various classes of plant and animals lectins that recognize sialic acids, comparing and contrasting the structural requirements and mechanisms involved in binding. Particular attention will be focussed on the recently evolving information about sialic acid recognition by certain C-type lectins (e.g., the selectins), I-type lectins (e.g., CD22 and sialoadhesin) and a complement regulatory protein (the H protein). The last two instances will provide examples of the significance of the side chain of sialic acids and its modification by 9-O-acetyl groups.

S2.3
O-Glycosylation and Tyrosine Sulfation Required for Recognition of the Glycoprotein Ligand by P-Selectin RICHARD D. CUMMINGS

Abstract: P-selectin is expressed by activated endothelium and platelets and is important in binding to granulocytes and monocytes in early steps of the inflammatory response. Recognition of leukocytes by P-selectin is dependent on a dimeric surface glycoprotein of apparent monomeric M_r~120 kD in leukocytes termed the P-selectin glycoprotein ligand-1 (PSGL-1). We have shown that a monoclonal antibody to PSGL-1 recognize peptide determinants at the extreme N-terminus and blocks adhesion to P-selectin. This N-terminal domain contains a number of Tyr residues occurring within a Tyr sulfation motif. Our recent structural and functional studies of PSGL-1 show that these Tyr are sulfated and that Tyr sulfation is critical for interactions of PSGL-1 with P-selectin. In addition, we have shown that PSGL-1 is a mucin-like glycoprotein which contains sialylated and fucosylated O-glycans that are required for binding to P-selectin. Interestingly, only a small percentage of the total O-glycans in PSGL-1 are fucosylated and these occur primarily on O-glycans containing a core-2 motif and an unusual extended type-2 polylactosamine structure with multiple α1,3-linked fucosyl residues in a sialyl Le^x and extended Le^x structure. This presentation will focus on recent developments in understanding the molecular recognition by P-selectin of the glycosulfopeptide found in at the extreme N-termius of PSGL-1.

S2.4
Interaction of peripheral lymph node dendritic cells and lymphocytes: involvement of galectins?

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Sequel to a recently studied L-selectin-induced aggregation among lymphocytes (V.V.R. Swarte et al, submitted), we wanted to know whether lymphocytes that are activated via L-selectin, and that do not have an altered binding capacity to the specialized High Endothelial Venules, perhaps show an altered binding to other cells or cell components present within the peripheral lymph node.

Therefore the binding capacity of non- versus L-selectin-activated lymphocytes to dendritic cells and macrophages was studied. It was shown that via L-selectin activated cells bound much better to dendritic cells than control cells did. We could not observe any change in binding of activated lymphocytes to macrophages.

The involvement of integrins and galectins in this enhanced binding is under current investigation. Furthermore, functional effects of the enhanced interaction of T cell and dendritic cells with respect to T cell activation and proliferation are now studied.

We propose that the activation of lymphocytes via L-selectin might result in an altered expression of cell surface molecules that can be important in the interaction of the cell with stromal elements in lymphoid organs, after extravasation has taken place.

S2.5
Effect of glycosaminoglicans on the KM^+-induced neutrophil haptotaxis. [1]GANIKO, L., [2]MARTINS, A. R., [3]ESPREAFICO, E. M. & [1]ROQUE-BARREIRA, M. C. *[1]Dep. Parasitology, Microbiology and Immunology, [2]Dep. Pharmacology, [3]Dep. Morphology, Faculty of Medicine of Ribeirão Preto, USP, Brazil.*

KM^+ is a lectin from *Artocarpus integrifolia* that induces neutrophil migration *in vitro* and *in vivo*. This chemoattractant activity was shown to be caused by haptotaxis rather than chemotaxis. The chemoattractant activity of KM^+ has been inhibited by D(+) mannose, both *in vivo* and *in vitro* (Santos-de-Oliveira et al., 1994), thus supporting the hypothesis that haptotaxis is triggered *in vivo* by the lectin binding site

interaction with a glycoconjugate located on the neutrophil surface or in the extracellular matrix. Intradermally injected (i.d.) $^{125}I.KM^+$ (200ng) has led to a selective staining of loose connective tissue and vascular endothelium. The radiolabelled area exhibited a maximum increase (5-fold) in neutrophil number 3 hours after injection, relative to i.d. 200ng ^{125}I-BSA. The effect of D(+) mannose and glycosaminoglycans on the binding of KM^+ to tissue elements has been studied using paraformaldehyde-fixed, paraffin-embedded, untreated rat skin. KM^+ was detected using an affinity-purified rabbit IgG anti-KM^+ antibody. Rabbit IgG was detected by an alkaline phosphatase based system. KM^+ binding to connective tissue and vascular endothelium was dose-dependent in the range of 1 to 10µg/ml. This lectin binding was inhibited by preincubating KM^+ with 0.4M D(+)mannose and was potentiated by heparan sulfate (50-500µg/ml). Chondroitin sulfate A (100µg/ml) showed no effect on binding. *In vitro* assays carried in a Boyden microchamber showed that 20µg/ml heparan sulfate potentiated (34%) the chemoattractant effect of 10µg KM^+. Chondroitin sulfate A did not change the chemoattractant activity of KM^+. Our results indicate that the carbohydrate recognition site is responsible for the binding of KM^+ to the connective tissue and the vascular endothelium. This binding is enhanced by heparan sulfate, which additionally potentiates the neutrophil chemotaxis induced by KM^+.

Financial support: CAPES, CNPq, FAPESP and FINEP.

S2.6

Binding of a branched *N*-acetyllactosamine-based glycosphingolipid to the *Erythrina corallodendron* lectin (ECorL) and the heat-labile toxin from *Escherichia coli* (pLT)

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Our recent findings that both the lectin (ECorL) from *Erythrina corallodendron* and the heat-labile toxin (pLT) from porcine *Escherichia coli* bind to paragloboside (Galβ4GlcNAcβ3Galβ4GlcβCer) as well as a branched *N*-acetyllactosamine-terminated six-sugar compound (Galβ4GlcNAcβ6(Galβ4GlcNAcβ3)Galβ4GlcβCer) are strongly suggestive of a common structural theme in their binding sites. Further investigations using the microtiter well assay and molecular modeling of the complexes of the six-sugar compund have been performed. It is found that the three-dimensional structures of the lectin and the toxin complexes reveals striking structural similarities around the Gal binding pocket despite lack of sequence and folding homology. The interactions of the penultimate GlcNAcβ3 in paragloboside differs, however, in the two cases. Whereas Arg-13 in pLT provides additional interactions with the hydroxymethyl group of GlcNAcβ3, the corresponding interactions in the ECorL complex are between the GlcNAcβ3 acetamido moiety and Gln-219. The branched six-sugar compound in both cases binds with β3-linked arm into the Gal binding pocket and with the β6-linked arm providing additional interactions on the protein surface which explain the enhanced affinity as compared to paragloboside.

S2.7

Recognition of glycosphingolipids by the neutrophil-activating protein of *Helicobacter pylori*

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A neutrophil-activating protein was recently isolated from the human gastroduodenal pathogen *Helicobacter pylori* . This 150 kDa protein induces an increased expression of CD11b/CD18 on neutrophils, and promotes the adhesion of neutrophils to endothelial cells.
Since the activation of human neutrophils might involve lectin-carbohydrate interactions, the binding of radiolabeled recombinant neutrophil-activating protein of *H. pylori* to glycosphingolipids from human neutrophils was investigated, using solid phase assays. A selective binding of the neutrophil-activating protein to four compounds in the acid glycosphingolipid fraction of human neutrophils was detected. By comparison of the binding preferences of the protein to reference glycosphingolipids from other sources, a minimum binding-active carbohydrate sequence, present in the acid glycoshingolipid fraction of human neutrophils, was identified.

SESSION 3: OLIGOSACCHARIDES AND PROTEIN RECOGNITION (PART II)
CHAIRPERSONS: ROBERT FELDMAN & GHISLAIN OPDENAKKER

S3.1

BIOSYNTHESIS OF SULFATED L-SELECTIN LIGANDS IN HUMAN HIGH ENDOTHELIAL VENULES (HEVs)

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High endothelial venules (HEVs) are specialized post-capillary venules found in lymphoid tissues, that support high levels of lymphocyte extravasation from the blood (1). Lymphocyte L-selectin plays a key role in the initial interaction of lymphocytes with HEVs *in vivo* by recognizing sulfated carbohydrate ligands on HEV mucin-like glycoproteins, GlyCAM-1, CD34 and MAdCAM-1. HEV-specific O-glycosylation of these three mucin-like proteins is required for high-affinity L-selectin binding. For example, CD34 is widely expressed in endothelial cells in most organs, but functions as an L-selectin counter-receptor only when appropriately decorated by HEV-specific sulfated oligosaccharides. Sulfation may be key to the uniqueness of the HEV ligands since a 6'-sulfated-sLe^X isoform has recently been identified as a major capping group of GlyCAM-1 and sulfation of both GlyCAM1 and CD34 has been shown to be required for high-affinity L-selectin binding and recognition by the HEV-specific monoclonal antibody MECA-79. The HEV

endothelium has been shown to be unique amongst vascular endothelium by virtue of its capacity to incorporate large amounts of radioactive sulfate. The requirement for sulfate in HEV ligands for L-selectin provides a functional basis for this metabolic specialization of the HEV endothelium.

To further characterize the molecular mechanisms involved in the biosynthesis of sulfated L-selectin ligands in HEVs, we have started to isolate genes that play a role in sulfate metabolism in HEVs. Studies with chlorate, a selective inhibitor of the synthesis of the high energy donor of sulfate PAPS (3'-phosphoadénosine 5'-phosphosulfate), had previously revealed that PAPS synthesis is required for sulfation of HEV ligands for L-selectin. Therefore, in order to isolate cDNAs encoding enzymes involved in PAPS synthesis in human HEVs, we screened an HEV cDNA library (2) by low stringency hybridization with heterologous probes. This strategy allowed us to isolate a novel cDNA encoding PAPS synthetase from human HEVs. The molecular characteristics of PAPS synthetase and its role in biosynthesis of sulfated L-selectin ligands in HEVs will be discussed.

(1) Girard, J.P. and Springer T.A. *Immunology Today* 1995, 16:449.
(2) Girard, J.P. and Springer T.A. *Immunity* 1995, 2:113.

S3.2

The role of endothelial sLex expression in lymphocyte extravasation

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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 analyzed with the in vitro Stamper-Woodruff binding assay. Concomitantly with the enhanced lymphocyte adhesion, the cardiac endothelium begins to de novo express sialyl Lea and sialyl Lex 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 significantly decreased either by treating the lymphocytes with anti-L-selectin antibody, or by treating the tissue sections with sialidase or anti-sialyl Lea or anti-sialyl Lex mAbs. Finally, we synthetized enzymatically several members of the sialyl Lex -family oligosaccharides and analyzed their ability to block lymphocyte adhesion to cardiac endothelium. Monovalent sialyl Lex (a tetramer), divalent sialyl Lex (a decamer) and tetravalent sialyl Lex (a 22-mer) all could significantly reduce lymphocyte binding, but the inhibition by tetravalent sialyl Lex -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 Lex family and had no effect on lymphocyte binding. These results suggests that L-selectin behaves as a "functional oligomer" on lymphocyte surfaces.

S3.3

Role of Lectin-Glycoconjugate Recognitions in Cell/ Cell Interactions Leading to Tissue Invasion

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Lectins are endogenous cell receptors, expressed in normal and transformed cells both circulating as well as in tissues. Their biological significance was shown in developmentally regulated processes of cell migration and embryonic maturation and differentiation. It has also been uncovered during various other normal and pathological processes.

This work will focus on the role of endogenous lectins and their glycoconjugate ligands in homing of circulating normal and cancer cells.

First, during the normal immune process of lymphocyte recirculation and journey of lymphocytes among the whole body through the secondary lymphoid organs in the search for antigens lectins are decisive molecules that allow the very first interaction which allows the arrest onto the endothelial cell layer.

Second, this led to the demonstration of a dual lectin-glycoconjugate interaction taking part in the initiation of the whole adhesion cascade between the adhering cell and the endothelial cell. This underlines the role of the endothelium which will be described here. Indeed, using high endothelial cell lines immortalized by us, we could demonstrate that the endothelium of microcapillaries is characterized by its tissue-specific properties along with a high microenvironment dependency. Both are decisive for selecting cells and allowing them to stop.

Third, such normal properties of endothelial cells and homing cells will be taken as an example and applied to understand pathologies like specific establishment of metastases in the case of colon cancer cells.

We shall finally, review the potential offered by lectins and the knowledge of their ligand structure to design efficient adhesion blockers or enhancers as invasion inhibitors or immunomodulators.

S3.4

CHARACTERIZATION OF OLIGOSACCHARIDES FROM AN ANTIGENIC MANNAN OF *SACCHAROMYCES CEREVISIAE.*

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IgG and IgA antibodies with specificity for *Saccharomyces cerevisiae* mannans have been described in serum from patients with Crohn's disease. An antigenically active homogeneous mannan has been isolated by gel filtration and the M_r determined to be 500 kDa by size exclusion chromatography on Superose 6 and 460 +/- 20 kDa by size exclusion chromatography/multi angle laser light scattering (SEC/MALLS; Young et al). In order to identify the antigenic sequences we have carried out acetolysis essentially as described by Natsuka et al. The method was modified by dissolving the sample in DMSO or DMF before addition of the peracetylation solvent (acetic acid-pyridine 1:1). Acetolysis products were analysed by labelling with 2-aminobenzamide and fractionation on Biogel P4 with fluorescence detection (Davies and Hounsell 1996). The method in which the antigen was dissolved in DMF before peracetylation gave the best yields and provided mono-, di- and tri saccharides making up more than 95% of the oligosaccharides. Their structures were determined by 1D and 2D TOCSY NMR.

References
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Natsuka S., Hase S. and Ikenaka T. (1987) *Anal. Biochem.* 167, 154
Davies M.J. and Hounsell E.F. (1996) *J. Chrom.* A 720, 227-33.

S3.5

Measurement of human neutrophil adhesion to platelets as a method to evaluate P-selectin dependent interactions. Foster, M.R., Priest, R.C., Malhotra, R. and M.Bird Glycobiology Unit, GlaxoWellcome Research and Development, Stevenage, Herts. UK., SG1 2NY.

A method using whole cells has been developed to evaluate potential inhibitors of P-selectin dependent adhesion. Previous methods have been described to study P-selectin function but have generally not studied interactions between platelets and neutrophils. Blood was taken by venepuncture from human volunteers, anticoagulated and the neutrophils and platelets isolated. Washed resuspended platelets were immobilised on 96-well plastic plates by centrifugation and fixed with 0.1% v/v formal saline. Immunostaining techniques showed P-selectin expression on the surface of these platelets similar in extent to that in the presence of stimulation by the thromboxane A_2 mimetic, U46619. After purification neutrophils were loaded with the fluorescent dye 2,7-bis-(2-carboxyethyl)-5-6-carboxyfluorescein (BCECF) and resuspended in a modified physiological salt solution containing 0.4mM Ca^{2+} (but no Mg^{2+}). BCECF-labelled neutrophils were incubated over the platelets (1hr, 4°C). The wells were washed gently (3 times) with physiological salt solution and finally the remaining adhered cells lysed with Triton-x-100 (2% v/v). Neutrophil adhesion was measured using a fluorescent plate reader (excitation 485nm, emmision 530nm). In the absence of inhibitors 17+/- 2% of those neutrophils added remained adhered after washing. An antibody to P-selectin (CLB-CD62, 0.2-10μg/ml) caused greater than 90% inhibition of neutrophil adhesion. In contrast, antibodies to E- (BBA.2) or L-selectin (LAM1-3) did not cause significant inhibition. Fucoidan (25-800μg/ml), an inhibitor of L- and P-selectin, inhibited neutrophil adhesion in a concentration-related manner, achieving complete inhibition at 400μg/ml. Similarly ethylenediaminetetracetic acid (EDTA, 10mM) caused extensive (>90%) inhibition of neutrophil adhesion. In conclusion, the method described measures neutrophil to platelet adhesion which appears to be selectin and Ca^{2+} dependent. Antibody studies indicate that P-selectin is essential for adhesion under these conditions.

S3.6

Lipids Isolated from Sheep Red Blood Cells Participate in the Rubino Reaction. ADEMILSON PANUNTO-CASTELO, *IGOR C. ALMEIDA & MARIA CRISTINA ROQUE-BARREIRA.
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The serum of some leprosy patients causes rapid sedimentation of formolized sheep erythrocytes, a phenomenon known as Rubino reaction, which is positive in forms of the disease that present impaired specific cell immunity for *Mycobacterium leprae*. We have identified the specific factor responsible for the Rubino reaction (Rubino factor - RF) as an IgM. The use of purified RF unambiguously demonstrated the requirement of a cofactor (CF) present even in normal serum for the occurrence of the Rubino reaction. In order to improve the sensitivity of the Rubino reaction, we tried to standardize an immunoenzimatic assay by coating the microplate well with monolayers of formolized sheep erythrocytes pre-treated with methanol. No reaction with the RF +CF was detected, suggesting that the erythrocytic ligand had been solubilized by methanol. Based on this hypothesis we evaluated the inhibition of the Rubino reaction with erythrocyte fractions, sequentially extracted with the organic mixtures chloroform:methanol (2:1), chloroform:methanol (1:2), chloroform:methanol:water (10:20:8) and 9% 1-butanol (v/v). Inhibition was observed with the chloroform:methanol (1:2) fraction. The direct binding of the RF+CF to those fractions was tested by chemiluminescent enzyme-linked immunosorbent assay. A positive and dose-dependent response was observed with the chloroform:methanol (1:2) fraction.After HPTLC in chloroform:methanol:$CaCl_2$ 0.2% (60:35:8) two bands of this fraction were rective with RF + CF, migrating at R_f 0.35 and R_f 0.89, respectively. RF and CF alone showed little or no reactivity with these bands. These results indicate that the ligands for the RF on the sheep erythrocyte surface are glycolipids and/or phospholipids. Purification and chemical characterization of these molecules are presently being carried out aiming the design of a more sensitive and specific method for diagnosis of leprosy.

S3.7

CD23 molecule, low affinity receptor for IgE, acts as a galactose-binding lectin

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CD23 molecule or FcεRII is a low-affinity receptor for IgE. It has no homology with other Fc receptors, but it showed a marked homology with Ca^{++}-dependent animal lectins. Recently, we have demonstrated that the CD23 molecule, indeed, interact with the galactose residue, especially with Gal-GalNAc- structure rather than Gal-GlcNAc of the terminal sugar chain of glycoproteins[1].

I also investigated the effects of glycosidases on aggregation and disaggregation of EBV-transformed B cell lines. The EBV-transformed cells (L-KT9) were separated into an aggregated-cell-rich fraction and a single-cell-rich fraction. Aggregated cells disaggregated after removal of galactose by β-galactosidase treatment, whereas single cells made large aggregation on sialidase treatment, and this aggregation was inhibited in the presence of asialo-fetuin. Using monoclonal antibodies, we showed that the aggregation occurs mainly through the interaction of CD23 as a lectin and galactose residue as its ligand. We also suggested that in the case of L-KT9 cells, the main ligand for CD23 will be other than CD21[2].

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S3.8

THE ROLE OF N-LINKED GLYCOSYLATION IN THE REGULATION OF ANTIGEN PROCESSING IN THE ENDOPLASMIC RETICULUM

*P.M.D. WOOD & T.J. ELLIOTT

Peptide epitopes, presented to cytotoxic T lymphocytes (CTL) in association with MHC Class I molecules, are generally derived from cytosolic and nuclear proteins. They are partially degraded in the cytosol by a multicatalytic complex known as the proteasome. The peptide fragments generated are transported by the TAP complex into the ER where they load onto Class I molecules. Cells which lack TAP are unable to present cytosolically-generated peptides to CTL. However, this defect can be circumvented by delivering antigenic fragments to the ER using an ER targeting signal. In addition, a 170 amino acid C-terminal fragment of influenza nucleoprotein targeted to the ER (L+IMP) can be processed to generate appropriate Class I-restricted epitopes. Thus enzymes capable of generating Class I epitopes from longer precursors exist inside the ER itself, and provide evidence for a complementary pathway for antigen presentation.
However, very few MHC Class I epitopes are derived from secretory pathway glycoproteins. Indeed, in contrast to L+IMP, full-length influenza nucleoprotein targeted to the ER (L+NP) is not processed to

generate the H-2Db restricted epitope in a TAP-deficient cell line (T2-Db). Unlike L+IMP, full-length NP contains two cryptic N-linked glycosylation sites that are both utilised. We have demonstrated that the glycosylated protein remains in the ER, suggesting that it is mis-folded. Despite this, it is long-lived and undergoes bi-phasic degradation with an initial lag-period. Treatment of transfected or Vaccinia-infected cells with the glycosylation inhibitor tunicamycin results in early rapid degradation of NP and restores the generation of the Db-restricted epitope within the ER of T2-Db. Use of the glucosidase inhibitor castanospermine produces similar results.

Our data suggests that N-linked glycosylation plays a role in protecting nascent proteins from the activity of ER proteases responsible for generating and trimming peptides for Class I loading and presentation. This could have implications for the pathogenesis of auto-immune disease where defective glycosylation may allow cryptic epitopes to be presented to the immune system.

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SESSION 4: OLIGOSACCHARIDES AND BIOLOGICAL FUNCTION
CHAIRPERSONS: FRANK HAY & HARRY SCHACHTER

S4.1

Reciprocity Of Phosphorylation And O-GlcNAcylation On Key Nuclear And Cytoplasmic Regulatory Proteins.

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The addition and removal of O-GlcNAc to nuclear and cytoplasmic proteins appears to be as abundant and as dynamic as the addition and removal of O-phosphate. Our laboratory is undertaking a multi-faceted approach to elucidate the roles of O-GlcNAcylation.

The microtubule associated (MAP) protein, tau, is heavily O-GlcNAcylated, suggesting a possible role for O-GlcNAc in Alzheimer's disease. Transfection of CHO cells with a cDNA encoding a cytosolic or a nuclear targeted form of galactosyltansferase, which are predicted to 'cap' O-GlcNAc moieties and prevent their cycling, appears to arrest cells in the cell cycle. Site mapping of casein kinase II indicates that it is O-GlcNAcylated adjacent to the CDC p^{34}-kinase sight that regulates its activity during the cell cycle. O-GlcNAc on the c-myc oncogene protein shows a reciprocal relationship with O-phosphate at threonine 58, the most important mutational 'hot spot' in human lymphomas. Studies of transcription indicate a direct role for O-GlcNAcylation in the assembly of the initiation complex at the promoter. An O-GlcNAc transferase from rat liver has been cloned, sequenced, and over-expressed in *E. coli*, baculovirus, and HEK 293 cells. The enzyme is highly conserved from rat to *C. elegans*. It has eleven tetratricopeptide (TPR) repeats which may be important in regulating the enzyme's intermolecular interactions. The O-GlcNAc transferase also is modified by tyrosine phosphorylation and O-GlcNAcylation. Subcellular distribution of the transferase changes dramatically when rat aortic smooth muscle cells are grown in hyperglycemic conditions. These different experimental paradigms are gradually yielding a coherent picture of O-GlcNAc's functions in many important cellular processes.(Supported by NIH CA42486, HD13563, Juvenile Diabetes Fdn, and The Mizutani Fdn.)

S4.2

Glycosylation of antibody molecules: *in vivo* and *in vitro*

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Glycosylation is a specific and controlled post-translational modification that influences multiple biological activities. Diseases and disease processes may be linked with aberrant glycosylation. Antibody molecules of the IgG class have a conserved glycosylation site at Asn-297 in the Fc region of the heavy chain. The oligosaccharide moiety is of the complex type, however multiple glycoforms are expressed.

Monoclonal antibodies, produced **in vivo** or **in vitro** have been shown to exhibit a unique, clonatypic, profile of glycoforms. The glycoform profile of antibody produced **in vitro** is influenced by the culture conditions and can be manipulated in a gross but not in a pre-selected manner. Differences in the efficiency of Fc receptor and complement activation have been reported for differing glycoforms.

In recent studies we have shown that:

i) The heavy chain isotype and allotype of IgG paraproteins influences β1-4 Gtase activity.
ii) The glycoform profile of paraproteins can vary over the course of disease, independent of the polyclonal component.
iii) Mutant heavy chains having a single amino acid replacement within the oligosaccharide interaction site can dramatically influence glycoform profiles and, consequently, biological activity.

For a review see: Jefferis, R. and Lund, J. (1996) Glycosylation of antibody molecules: structural and functional significance. In: Antibody Engineering. Ed. JD Capra. Karger, Basel.

S4.3

Heparin Potentiates Neutrophil Migration Induced by IL-8 in vivo

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Chemokine IL-8 has a chemoattractant effect on neutrophils by haptotaxis, made possible by its interaction with proteoglycans of the extracellular matrix . In vitro assays have demonstrated that heparan sulfate, but not heparin, potentiates the chemoattraction exerted by IL-8. In the present study we evaluated in vivo the interference of heparan sulfate or heparin with the neutrophil migration induced by IL-8. Purified rat IL-8 activity (4.15 μm/animal) preincubated with heparan sulfate (50 μg/animal) or heparin (77 μg/animal) was assayed on the dorsal air pouch. Only heparin was found to interfere with the chemokine activity by potentiating it by 118%. We then investigated the relation between this observation and that reported by others, that IL-8-induced migration depends on the presence of mast cells, cells containing heparin-rich granules. We studied the neutrophil exudation induced by IL-8 (4.14 μg/animal) into the peritoneal cavity of rats previously depleted of the intracavity mast cell populations. In these animals, neutrophil migration was reduced by 32% when compared to that observed in normal animals. The response of depleted rats was reconstituted by preincubation of IL-8 with heparin (77 μg/animal),

reaching normal levels. The potentiating activity of heparin on neutrophil migration may be attributed to the ability of glycosaminoglycan to induce the expression of heparan sulfate, a component that may permit the occurrence of the haptotactic gradient of IL-8 which directs the movement of neutrophils migrating from the postcapillary venule. We suggest that heparin released by cytoplasmic granules may be responsible for the participation of mast cells in IL-8 induced neutrophil migration.

S4.4

The role of human IgG glycans in the binding of C1q component of human complement.

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It's now well-known that intercellular, intermolecular and cell-molecule interactions are mediated by carbohydrate-protein recognition. The aim of this study was to evaluate accessibility of IgG glycans and their role in C1q binding. Monomeric IgG (mIgG) from healthy donors, mIgG from rheumatoid arthritis (RA) patients (1.78 and 1.25 galactose residues per one carbohydrate chain respectively) and model immune complexes (MIC) of wide range of molecular masses (MM) prepared by heating at 63°C or cross-linking by glutaraldehyde of normal mIgG were taken into work. The efficacy of C1q binding was determined by hemolytical assay and ELISA. The specific binding C1q with oligosaccharides was confirmed by lactose interaction inhibition. Dissociation constants for normal mIgG and IgG from RA patients reliably differed (29.0±3.5 and 47±4.0 mmM respectively, $p<0.01$). Lactose inhibited C1q binding with mIgG and MIC in dose-dependent manner, maximal inhibition was achieved at 0.1 M lactose (30%).Saccharose didn't influence on C1q binding with IgG. Estimation free energy increase of binding C1q with IgG of different MM showed that only two molecules of IgG are involved in the interaction with C1q molecule.

Thus, IgG glycans are accessible for binding with high molecular weight ligand - C1q. Galactose-containing IgG determinants take part in C1q binding, but they aren't the main site of this interaction. Increase of local density of galactose determinants, not the changes in their accessibility during MIC formation is the most important factor for effective C1q binding.

S4.5

Cellular and ultrastructural localisation of the Forssman antigen, a possible xenoantigen, in mouse vessels

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Antibodies against the Forssman antigen, a five-sugar glycolipid, are associated with hyperacute xenorejection in the mouse heart to rat xenotransplantation model. Previous light microscopic studies in the literature claim that the Forssman antigen is expressed on mouse endothelium. This work was undertaken to determine the precise localisation of the Forssman antigen in mouse vascular tissue.

Cell culture: A microvessel enriched suspension from mouse hearts was prepared by density centrifugation using Percoll®. Endothelial cell primary cultures, short time (48 hrs) as well as long time cultures (>3 weeks), were incubated with monoclonal anti-Forssman antibody. Binding was visualised with FITC conjugated secondary antibodies. Microvessel endothelial cell origin was determined by morphological criteria and uptake of DiI labelled AcLDL. All endothelial cells were completely negative for the anti-Forssman antibody. A minor cell population, without endothelial cell characteristics, was however strongly positive for the anti-Forssman antibody. The positive cell population had a conspicuous dendritic morphology.

Electron microscopy: Mouse hearts were perfusion fixed. Vibratome sections were made and incubated with the anti-Forssman antibody. Biotinylated secondary anti-rat Ig antibody and streptavidin conjugated gold particles were then added. After silver enhancement final sections were made by an ultramicrotome. The endothelium did not stain with the anti-Forssman antibody. The antibody did however stain a macrophage like cell localised adjacent to, but outside of, the endothelial cell. The findings were supported by immunofluorescence light microscopy of cryostat sections. Control incubations with normal rat serum were negative for all staining procedures.

Conclusion: The main expression of the Forssman antigen in mouse heart capillary is on the plasma membrane of a macrophage like cell with dendritic morphology localised adjacent to the endothelial cells.

S4.6

GLYCAN-GLYCAN BINDING STRENGTH BETWEEN *GLYCONECTIN* CELL ADHESION PROTEOGLYCANS MEASURED BY ATOMIC FORCE MICROSCOPY

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Our investigations in invertebrate and mammalian models provided evidence that a new molecular mechanism of polyvalent and specific carbohydrate-carbohydrate binding between proteoglycans can mediate cell adhesion. Recent partial sequencing of adhesion glycan structures and cloning of the gene for invertebrate proteoglycan core protein indicated novel carbohydrate and protein structures. The ability of this new class of proteoglycans to connect cells via glycan-glycan interactions led us to name them glyconectins. We have developed atomic force microscopy (AFM) technology to direct measure intermolecular binding strength between individual pairs of ligand and receptor molecules in physiological solution. Homophilic glyconectin interactions were investigated by AFM after covalent attachment of the protein core to the AFM sensor tip and to a flat surface leaving functional carbohydrates unmodified. The functionalized cantilever tip was moved in contact with the glyconectin modified surface and then retracted. The hysteresis of the lever is the direct measure of the intermolecular adhesion forces in piconewtons. Such direct measurements of binding strength intrinsic to cell adhesion molecules are necessary to assess their contribution to the maintenance of the anatomical integrity of multicellular organisms. Until now the forces for any type of intermolecular interactions have remained unknown. AFM measurements of the binding strength between glyconectin cell adhesion proteoglycans indicated that one pair of molecules could theoretically hold the weight of 1,600 cells in physiological solution. These results provided the first essential and quantitative evidence that carbohydrate-carbohydrate binding can perform the adhesion function that we have assigned to it.

S4.7
L-SELECTIN-MEDIATED SIGNALING AND ACTIVATION OF NEUTROPHILS

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It is known that L-selectin binds to glycoconjugates containing the tetrasaccharide sialyl Lewis X in a Ca^{2+}-dependent manner.In addition, a number of other acidic oligosaccharides (for example heparin or chondroitin sulphate) or glycolipids (for example sulphatides) bind to L-selectin independent of cations. Earlier we have reported that L-selectin binds to charged phospholipids, such as cardiolipin and phosphatidylserine, but not to neutral phospholipids such as phosphatidylcholine [Malhotra et al., (1996) Biochem J. **314**, 297-303]. No interaction between E-selectin and any phospholipid was observed. Based on the binding characteristics, sequence analysis and structural modelling of L-selectin, we suggested that the amino acid sequence KKNKED (residues 84-89) is a novel site for the binding of acidic species to L-selectin. This motif localises close to the putative carbohydrate binding domain of L-selectin and may be a second site within the lectin domain for the interaction of leukocyte L-selectin with its natural endothelial ligands. Herein, we show that binding of L-selectin ligands, via cardiolipin binding site, to neutrophils results in intracellular signaling and superoxide production, indicating that the binding of physiologically relavent ligands to L-selectin via the amino acid sequence KKNKED (residues 84-89) can mediate both binding and activation of human neutrophils.

SESSION 5: GLYCOSYLATION AND INFLAMMATION
CHAIRPERSONS: DIRK VAN DEN EIJNDEN & JOEL SHAPER

S5.1
Immunodetection of α1,3Fucosyltransferase VI

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The most common selectin ligand sialyl-Lewis^x (Slex) contains α1,3fucose transferred by one of 5 different α1,3fucosyltransferases (FucT), designated fucT III to VII. Little is known on tissular and subcellular localization of these enzymes. To approach these problems we initiated a project on immunolocalization and trafficking studies on fucTV and fucTVI.

A polyclonal antiserum to rec. fucTVI was obtained and shown to cross-react strongly with fucTV, little with fucTIII but not with fucTIV. Heterologous expression of fucTVI showed typical Golgi localization. Immunoprecipitation of FucTVI yielded 3 forms of 43, 48 and 51 kD and their dimers, all of them sensitive to Endo H and PNG-ase F. The antibody also reacted with a Golgi-associated fucT in HepG2 cells.

Monospecific antibodies to both fucTV and fucTVI were raised to peptides with lowest homology. The peptide antiserum to fucTV was shown to be monospecific. The peptide antiserum to fucTVI only identified ER-associated forms of rec fucTVI while affording the identical banding pattern after immunoprecipitation as the anti-fucTVI antiserum.

The antipeptide antiserum to fucTVI strongly stained Weibel-Palade (WP) bodies in human umbilical cord endothelial cells (HUVECs) as shown by co-localization with either P-selectin or von Willebrand factor, while the antiserum to fucTVI afforded a typical Golgi localization. Work is in progress to identify the crossreacting, fucTVI-like material in WP bodies. These findings confirm the presence of fucTVI as a Golgi enzyme in liver and suggest that in endothelial cells it exerts another function possibly related to exteriorization of WP bodies.

Supported by grants 3100-40749.94 of the Swiss National Science Foundation to EGB and 2305.1 of the Kommission für Technologie und Innovation to EGB.

S5.2
Cytokine Glycosylation: Biological Role of N- and O-linked Sugars

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The interaction of cytokines with cytokine-receptors and the induced signaling cascades depend on both the protein and the attached carbohydrates of the ligand and its receptor. Structural heterogeneity of cytokines is achieved by gene duplication or by glycosylation: usually multigene cytokine families (e.g. alfa-interferons, chemokines) do not contain N-linked sugars, but may carry O-links, whereas single copy cytokine genes encode glycoproteins with one or more N-linked sugars (1). Particular cytokines (e.g. interleukin-1) for which sensitive assay systems have been developed, have been used to probe the effect of sugars on receptor interactions and on signal transduction and cell activation. Whereas larger N-linked structures seem to down-modulate the specific activity of cytokines, the effects of O-linked sugars are less documented. Some cytokines (e.g. TNF-α) possess lectin-like domains, which may influence the interaction with carbohydrates of receptors and of host extracellular matrix molecules or of parasite sugars (2). Possibly these lectin domains might influence oligomerization of cytokines which is important for the interaction with hetero- or multimeric cytokine receptors

(1) FASEB J. (1995) **9**: 453–457.
(2) Science (1994) **263**: 814–817.

S5.3

Occurrence and possible function of inflammation-induced expression of sialyl Lewis-X on acute-phase proteins

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Acute and chronic inflammatory conditions induce the expression of (sialyl) Lewis-X determinants ((S)LEX) on at least three acute phase proteins, alpha-1-acid glycoprotein (AGP), alpha-1-protease inhibitor (API) and alpha-1-antichymotrypsin (ACT). The level of SLEX expression is the most pronounced on AGP, most probably because it contains in addition to di- and triantennary glycans also tetra-antennary glycans, which are absent from API and ACT.
The hepatic fucosyltransferase VI has appeared to be responsible for the LEX expression on these glycoproteins. Hormones like estrogen induce a decrease in the expression of (S)LEX on all three proteins under normal as well as inflammatory conditions. Glycoforms of AGP containing high or low amounts of alpha(1-3)-linked fucose and SLEX groups and differing in degree of branching were isolated from patient and normal sera. Differences in immunomodulatory properties of these glycoforms were assayed for in various model systems. The results of these studies will be discussed.

S5.4

SEQUENTIAL STUDY OF SERUM GLYCOPROTEIN FUCOSYLATION IN ACUTE HEPATITIS

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Background: α-Fetoprotein is a useful diagnostic marker in hepatocellular carcinoma, during which its serum level increases and its glycan structure is hyperfucosylated. Normally expressed glycoproteins (α1-antitrypsin and transferrin) are also hyperfucosylated in hepatocellular carcinoma. α-fetoprotein serum levels are also increased in conditions associated with hepatic regeneration, such as acute hepatitis. We conducted a longitudinal study of the α1-6 fucosylation pattern of serum α-fetoprotein in 10 patients with acute hepatitis and compared it to that of transferrin and α1-antitrypsin.
Methods: Protein levels were measured by using immunochemical assays. Crossed affinoimmunoelectrophoresis in the presence of *Lens culinaris* agglutinin was performed for each protein, and the fucosylation index, corresponding to the agglutinin reactive fraction, was determined. The results were compared to those in 25 healthy donors and 5 newborns.
Results: α-fetoprotein was hyperfucosylated and remained stable throughout the course of the disease. In contrast, serum transferrin and α1-antitrypsin gradually became hyperfucosylated during the course of acute hepatitis. The transferrin and α1-antitrypsin fucosylation indexes correlated with each other but not with the α-fetoprotein fucosylation index. No correlation was found between α-fetoprotein, α1-antitrypsin and transferrin fucosylation indexes and the corresponding glycoprotein serum levels.
Conclusion: Hyperfucosylation of α-fetoprotein is not specific of hepatocellular carcinoma. Increased α1-6 fucosylation should not be considered solely as a tumor marker, but might also reflect cell proliferation. The study of α1-6 hyperfucosylation process of normally expressed glycoproteins awaits further investigation, to test its usefulness as a new marker of liver regeneration during the follow-up of acute hepatitis.

S5.5

Preferential binding of influenza A and Sendai virus to fucosylated polylactosamine gangliosides

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Influenza A and Sendai viruses are known to bind to various extent to neolacto-series gangliosides IV^{3}Neu5Ac-nLcOse$_4$Cer, IV^{6}Neu5Ac-nLcOse$_4$Cer and VI^{3}Neu5Ac-nLcOse$_6$Cer, which are the dominant gangliosides of human granulocytes. Recently, minor gangliosides of granulocytes were characterized and found to express sialyl Lewis^x and VIM-2 epitopes. These long chain linear monosialogangliosides with nLcOse$_8$- and nLcOse$_{10}$-cores, carrying one to three fucoses, are shown in this study to bind with strong avidity to influenza A/PR/8/34 (H1N1), A/X-31 (H3N2) and Sendai virus (Z-strain) using the overlay technique. These and recent data from other groups imply that selectins and virus hemagglutinins are capable to compete with lipid bound sialyl Lewis^x and VIM-2 epitopes on myeloid cells during inflammatory reactions.

S5.6

Changes in concentration and glycosylation of two acute phase proteins in major depression.

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There is now strong evidence that affective disorders may be accompanied by abnormal immune activation. We have studied changes in plasma concentration of three positive acute phase proteins (apps): C-reactive protein (CRP), alpha-1-acid glycoprotein (AGP), alpha-1-antychymptripsin (ACT) and major microheterogeneity of AGP and ACT in 141 patients with major depression disorder and in 20 age- and sex mached controls. The changes in major microheterogeneity of AGP and ACT were expressed as reactivity coefficients (AGP-RC, ACT-RC). Also measured were the levels of interleukin-6 (IL-6) and its soluble receptor (sIL-6R) and monocyte count. There were 81 inpatients from Department of Adult Psychiatry in Poznan and 60 outpatients from Depression Research Unit in Philadelphia. Diagnosis was assessed acording to DSM IV and ICD-10 criteria: all patients were diagnoses as major depression. Concentration of apps were measured by rocket immuno-electrophoresis and reactivity coefficient (RC) of their microhetergeneity by crossed-affinity electrophoresis (CAIE).
The results obtained showed that two third of depressed patients studied, exhibited during acute depressive episode some immune disturbances: one with high AGP, ACT and high AGP-RC and second with high AGP,ACT and low AGP-RC. Patients with highest AGP-RC

and higher AGPand ACT values had longer duration of the illness and of the studied depressive episode, were treatment resistant and had higher monocyte count and concentrations of IL-6.
The character of changes in microheterogeneity of AGP bears some similarity to these observed in other diseases with immunological disturbances.

S5.7

Glycosylation of α1-acid glycoprotein in inflammatory disease: analysis by high-pH anion-exchange chromatography and concanavalin A crossed affinity immunoelectrophoresis

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Abstract
High-pH anion-exchange chromatography with pulsed amperometric detection is a highly sensitive technique that can be used for detecting changes in sialylation and fucosylation, as well as different branching patterns of N-linked oligosaccharides in glycoproteins. We examined the N-glycans of α1-acid glycoprotein obtained from seven patients with various inflammatory conditions with this technique, as well as traditional concanavalin A crossed affinity immunoelectrophoresis. We found the chromatographic profiles to be characteristic for each group of patients, and conclude that this technique can be recommended for mapping glycosylation changes in α1-acid glycoprotein that may be specific for a certain type of inflammation. We aim to further investigate the clinical use of glycosylation profiles of α1-acid glycoprotein in patient samples, and also to develop methods more suitable for routine analysis.

S5.8

Changes in α3-fucosylation of Human Serum Acute-Phase Glycoproteins in Inflammation and Oral Estrogen Treatment: Role of Fucosyltransferase-VI

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The α3-fucosylation of α_1-acid glycoprotein (AGP) increases in inflammation and decreases during oral estrogen treatment. The same changes were found for α_1-antichymotrypsin (ACT) and α_1-protease inhibitor (PI). The major α3-fucosyltransferase (α3-FT) activity in human plasma is encoded by the gene for FucT-VI (FUT6). A missense mutation (G739 → A) in this gene is responsible for the deficiency of α3-FT activity in plasma. To examine whether this α3-FT is the sole enzyme responsible for the α3-fucosylation of serum glycoproteins in the liver, we studied the fucosylation of three glycoproteins in sera of individuals with or without inactivated FUT3 and/or FUT6 gene(s), but with a functional FUT5 gene. AGP was used as the principal reporter protein for liver α3-FT activity, because of its high fucose content. No fucosylation of AGP was found in the individuals lacking FucT-VI activity, in control and inflammatory conditions. This AGP was not intrinsically resistant to fucosylation, since it was susceptible to *in vitro* fucosylation using an α3/4-FT isolated from human milk. The same was found for ACT and PI. In all individuals with α3-FT activity in plasma the degree of fucosylation of AGP was correlated with α3-FT activity (Rs = 0.82). These data indicate that FucT-VI is responsible for the α3-fucosylation of serum glycoproteins produced in liver.

SESSION 6: GLYCOSYLATION AND DISEASE (PART I): IMMUNOGLOBULINS
CHAIRPERSONS: STEVE HOMANS & GERRY HART

S6.1

The Glycosylation of the Complement Regulatory Protein, Human Erythrocyte CD59

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Lysis of invading micro-organisms by the membrane attack complex of the complement system is an integral part of the innate immune response. Complement activation involves powerful effector mechanisms against which host cells are protected by inhibitory proteins. CD59, a cell surface glycoprotein with a glycosylphosphatidylinositol (GPI) anchor, binds the complement proteins C8 and/or C9 in the activated membrane attack complex, preventing the formation of the fully developed complex. CD59 contains all three types of post-translational modification known to involve glycosylation (a glycan anchor, N-linked and O-linked glycans). This investigation gives an overview of all the major structural features of CD59, making it the first cell surface glycoprotein to be defined in detail. First, the GPI anchor was shown to consist mostly of the minimum core glycan sequence Mana1-2Mana1-6Mana1-4GlcN linked to a phosphatidylinositol (PI) moiety with the structure sn-1-O-alkyl-2-O-acylglycerol-3-phospho-1-(2-O-palmitoyl) myo-inositol. The alkyl group was a mixture of C18:0 and C18:1 chains and the acyl group attached to the glycerol backbone was exclusively a C20:4 fatty acid. The presence of the palmitoyl (C16:0) fatty acid substituent on the 2-hydroxyl of the myo-inositol ring is consistent with the resistance of CD59 to the action of bacterial PI-specific phospholipase C. The CD59 GPI anchor is essentially identical in structure to that described for human erythrocyte cholinesterase (Deeg et al., (1992) J. Biol. Chem. 267, 18573-18580), suggesting that human reticulocytes produce only one type of GPI anchor structure. This is the first comparison of GPI anchor structures from different proteins expressed in the same tissue. Second, human erythrocyte CD59 has one highly conserved N-linked glycosylation site at Asn 18. MS analysis of the desialylated N- glycans revealed over 130 N-linked oligosaccharides with different compositions, 30% of which contained outer arm fucose. The glycans consisted of families of bi-, tri- and tetra-antennary complex-type structures with and without lactosamine extensions. The oligosaccharides were characterised using a novel predictive HPLC

methodology. Using a new approach to sequencing the total pool of fluorescently labelled oligosaccharides was simultaneously digested with multiple enzyme arrays and the products analysed by HPLC. Third, approximately 30% of the total glycans were sialylated O-linked sugars. The predominant asialo structures were the disaccharide Galb1-3GalNAc and the monosaccharide GalNAc. Molecular modelling suggested several possible roles for the heterogeneous array of glycans. These included orienting the active site (defined by site directed mutagenesis: Bodian, D.L., Davis, S.J., Rushmere, N.K. and Morgan, B.P. - in preparation) while protecting it from protease cleavage. In addition the sugars may limit the interactions of the protein with other CD59 molecules and with the cell surface.

S6.2

Glycosylation & Autoimmune Disease

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Oligosaccharides are more than a decorative irrelevance when it comes to molecular mechanisms of disease. The last decade has witnessed an exponential interest in the associations of oligosaccharides with disease mechanisms, and significant advances have been made in the design of carbohydrate based therapies and diagnostic techniques. Some essential facts are as follows:

Oligosaccharides have functional potential. They are able to form a variety of branched and linear arrays, which not only increase their own heterogeneity, but the proteins to which they attach.

Oligosaccharide synthesis is precise. This occurs within the endoplasmic reticulum and golgi and the exact characteristic of the carbohydrate product is under exquisite control. Changes in the oligosaccharide profile of a protein therefore does not occur at random.

Oligosaccharides are associated with arthritis. In rheumatoid arthritis the **galactose** content within immunoglobulin G is reduced. A synthetic abnormality involving the lymphocytic enzyme **galactosyltransferase** may be responsible. This enzyme is reduced in both B and T lymphocytes in patients with rheumatoid arthritis.

Agalactosyl-IgG has a pathogenic role. Some IgM ***rheumatoid factors***, derived from synovial tissue, are dependent on the lack of galactose within IgG for their binding. ***Mannose binding protein*** interacts with N-acetylglucosamine bearing structures. Agalactosyl-IgG is such a structure and when bound by mannose binding protein complement activation may result. This is thought to trigger an inflammatory process. ***Animal models of arthritis*** have been developed in which the inflammatory process is triggered by agalactosyl-IgG.

Galactosyltransferase. Glycosylation homeostasis within rheumatoid arthritis lymphocytes is thought to be abnormal. In healthy individuals there is a positive feedback mechanism ensuring that galactosyltransferase levels increase as IgG galactose levels reduce. In rheumatoid arthritis this does not occur.

In what way could enzymatic control be abnormal? In serum, there are two peaks of enzymatic activity after isoelectric focusing. In rheumatoid arthritis, in contrast to psoriatic arthritis and other rheumatic diseases and healthy individuals, there is a significant acidic shift in the galactosyltransferase activity profile, with a unique peak forming a pH 5.05. It is thought that these isoenzymes may reflect a reduced efficiency in ability to galactosylate structures such as immunoglobulin G.

Are the rheumatoid arthritis associated glycosylation abnormalities unique? The answer to this is probably no. Similar changes have been shown to occur in a number of other diseases, but detailed examination reveals that unique IgG carbohydrate profiles are associated with each rheumatic disease. For example, psoriatic arthritis may be associated in a similar manner as rheumatoid arthritis with agalactosyl-IgG but, in addition to this, sialylated oligosaccharides are significantly increased on the IgG molecule.

Immunoglobulin sugar printing is therefore possible whereby individual rheumatic diseases have been shown to be associated with a specific carbohydrate profile. It is now important to test these preliminary data in larger studies to determine whether they will aid diagnosis and perhaps enable further dissection of disease mechanism.

S6.3

DEFECTIVE IgA1 GLYCOSYLATION IN IgA NEPHROPATHY

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IgA nephropathy (IgAN) is a common type of glomerulonephritis characterised by deposition of polymeric IgA1 (plgA1) in the glomerulus and IgA1 immune hyperactivity. There is little evidence that immune complex deposition is the prime cause of the IgA1 deposition. Abnormal IgA glycosylation is a candidate abnormality which may predispose to glomerular plgA1 deposition.

The hinge region of IgA1 is unique among circulating immunoglobulins for its O-glycans linked to serine: **Serine - α1,3 - GalNAc - ß1,3 - Gal**. Alterations in these glycans are likely to have significant effects on the interactions of IgA1 with receptors and other effector pathways; for example the ligand for the asialoglycoprotein receptor (the main clearance mechanism for circulating IgA1) is hinge region galactose, and the recognition site for Fc alpa receptors (expressed on leucocytes and glomerular mesangial cells) is also close to the hinge.

Using lectins specific for N-acetylgalactosamine (GalNAc) we have shown increased binding of serum IgA1 in IgAN consistent with increased expression of GalNAc or lack of terminal Gal. The same pattern is seen in serum IgA1 in Henoch-Schönlein purpura (another IgA related disease) but only when there is renal involvement.

ß,1-3 galactosyltransferase, the enzyme required for galactosylation of Gal NAc, has reduced activity in peripheral blood B cells in IgAN and enzyme activity correlates with altered glycosylation defined by *Vicia villosa* binding.

Others have reported abnormal sialylation of serum IgA1 in IgAN which alters IgA1 aggregability *in vitro.*

Further molecular definition of these O-glycan and enzyme abnormalities is required. However, these defects may represent a fundamental pathogenic abnormality in this common renal disease.

S6.4

The role of changes in IgG glycosylation in rheumatic diseases

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Changes in IgG glycosylation are a consistent feature of patients with rheumatoid arthritis. Alterations are also seen in other rheumatic diseases as well as certain non-rheumatic inflammatory or infectious situations. The precise sugar structures present vary in a disease-specific fashion; for instance, in rheumatoid arthritis, juvenile onset chronic arthritis and Crohn's disease, galactose and N-acetylglucosamine exhibit a reciprocal relationship, whereas in Sjögren's syndrome an increase in galactose is associated with a parallel increase in N-acetylglucosamine. These changes are being analysed more closely by separately examining Fc and Fab regions to

determine whether differential glycosylation occurs in the various parts of the molecule. These structural studies are being examined in relation to disease activity and pattern and early results indicate that sugar changes are related to activity in rheumatoid arthritis.

Our studies show that certain monoclonal rheumatoid factors bind better to agalactosyl IgG. This is being related to disease activity by looking at the specificity and affinity of polyclonal rheumatoid factors for different IgG glycoforms in various disease states.

We have found that immune complexes from patients with rheumatoid arthritis are particularly rich in agalactosyl IgG compared with non-complexed IgG. This leads us to suggest that the production of agalactosyl IgG may be linked to particular antibody specificities rather than being a property of all B-cells in a patient. To investigate this further we are looking at the glycosylation of isolated autoantibodies from patients with a range of autoimmune diseases and at specific antibodies raised following immunisation with vaccine antigens.

S6.5

Fab and Fc glycosylation in rheumatoid arthritis, Sjögren's syndrome and normal IgG

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Galactose and N-acetylglucosamine (GlcNAc) on IgG from patients with rheumatoid arthritis are inversely related. This is a direct indication that as the level of galactose decreases, there is an increase in the level of the next sugar along the oligosaccharide chain, GlcNAc.

However in Sjögren's syndrome, we have shown galactose and GlcNAc to have a slight positive association arising from parallel changes in both sugars. This positive association can be explained in one of three ways: an increase in the overall glycosylation, differential glycosylation or variations in the level of bisecting GlcNAc. Increased overall glycosylation could be influenced by the presence of other glycosylation sites in addition to the one in the Fc region, and would be dependent on the presence of the peptide glycosylation motif Asn-X-Ser/Thr in the Fab domain. As differential glycosylation has been demonstrated in other glycoproteins and could be due to variations resulting from differential processing of the Fab sugars, it would be possible to have unaltered Fc sugars while Fab sugars vary. Finally, as bisecting GlcNAc is attached to the C4 position of the b-mannosyl residue between the bi-antennary oligosaccharide arms, exposed bisecting GlcNAc may be detected while, at the same time, terminal GlcNAc could be masked by galactose, thus maintaining apparent constancy in GlcNAc expression.

We have analysed IgG Fab and Fc glycosylation in healthy individuals as well as rheumatoid arthritis and Sjögren's syndrome patients in an attempt to understand more fully, our previous findings of a positive association between galactose and GlcNAc levels on Sjögren's syndrome IgG.

S6.6

FACE analysis of the spectrum of oligosaccharides associated with total serum glycoproteins in Rheumatoid arthritis

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We have previously demonstrated the applicability of fluorophore assisted carbohydrate electrophoresis (FACE) in the analysis of rheumatoid arthritis (RA) associated IgG oligosaccharide changes.
In this study, we have investigated the use of this technique to examine the range and type of oligosaccharides associated with total serum glycoproteins. Serum glycoproteins from RA (n=6) and age-matched healthy individuals (n=4) were digested with peptide-*N*-glycanase F and the oligosaccharides released, coupled with fluorophore 8-aminoaphthalene-1,3,6-trisulphonic acid. Densitometric analysis of the 6 bands obtained from the electrophoretic separation of the labelled oligosaccharides, revealed a significant increase in the relative intensity of band 4 in the RA group when compared with healthy controls (Mean % intensity± SEM: RA; 32.3±1.3 vs control; 25.0±0.4, 2p=0.003). This band has been shown to co-migrate with, and thus represent, the agalactosylated *N*-linked complex biantennary oligosaccharide structures, which are a perpetual feature of RA IgG and a well established biochemical marker for this disease.
To determine whether the observed increase in the relative proportions of these agalactosylated structures in RA serum are i) simply an indirect measure of increased incidence of hypogalactosylated IgG, or ii) a reflection of an overall shift in galactosylation, which encompasses other glycoproteins, we are currently investigating the FACE profile of paired RA sera and its constituent IgG.

S6.7

STRUCTURAL AND FUNCTIONAL STUDIES IN MAN OF A POLYSACCHARIDE ANTIGEN OF THE YEAST *SACCHAROMYCES CEREVISIAE*

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Data accumulated over the last several years have demonstrated that a heat-stable, soluble antigen of *Saccharomyces cerevisiae* (Sacc) is immunogenic in man and, in particular, IgA anti-Sacc antibody discriminates Crohn's disease from ulcerative colitis. Furthermore, Sacc has been shown to induce cellular proliferative responses *in vitro* which appear to be predominantly mediated by T cells (Darroch *et al. Immunology* 1994, 81, 247-252) thus providing an approach to Sacc antigen-specific T cell cloning by limiting dilution. Structural studies to date indicate that the major antigenic moiety of Sacc, based on competitive inhibition of IgG and IgA antibody binding, comprises 90% mannose: 10% protein (total Mr 500 kDa) with the mannose residues connected by 1→2 and 1→3 linkages (Young *et al. Carbohydrate Polymers* 1996 in press). Taken together, these experimental approaches provide a basis for further elucidation of the antigenicity and immunological properties of a novel ubiquitous fungal antigen.

S6.8

Decreased binding of serum immunoglobulin M to tumour-associated carbohydrate antigens in patients with breast cancer.

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The Thomson-Friedenreich antigen T, its precursor Tn and the mucin-associated disaccharide sialylated Tn (STn) are expressed on the majority of cancers, with limited or no expresssion on corresponding normal tissues. In contrast to the T antigen, the expression of Tn and STn by tumours has been associated with disease progression. Serum anti-carbohydrate antibodies to these types of blood group related antigens are known to exist in the normal healthy population possibly as a result of cross-reactivity to gut bacteria. Given this, alterations in glycosylation on breast cancers should be recognized and an immune response generated. The aim of this study was to investigate whether there is a decreased immunity to tumour-associated carbohydrate antigens in patients with breast cancer and whether this is associated with disease progression.

Using adapted ELISA techniques, serum levels of IgM have been measured which bind to Tn and STn in normal healthy controls, patients with benign breast disease and 89 patients with breast cancer. The levels of circulating serum and complexed IgM have also been measured in these individuals using ELISA and polyethylene glycol precipitation followed by single radial immuno-diffusion respectively.

The results indicate decreased binding of serum immunoglobulin M to both Tn and STn in patients with breast cancer when compared to normal healthy controls and patients with benign breast disease. These decreases do not appear to be associated with serum levels of circulating immunoglobulins or immune-complexes.

S6.9

Functional activity (FA) of complement components (CC) and degalactosylated IgG in rheumatoid arthritis (RA) patients.

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Deficiency of IgG galactosylation in RA patients is widely described, but consequences of this phenomenon are under investigation.

We have studied 12 RA patients with confirmed diagnosis (ARA criteria, 1988), all women from 28 to 64 years old with high disease activity. 42 healthy age-matched women were in control group. Galactose content in IgG samples was determined by HPGLC, FA of CC (C1q, C1-C5, CH50) - by microhemolytical assay, other test were made by routine techniques.

We have found all RA patients had significant increase of FA of CC and C3 and C4 levels compared with donors ($p<0.001$). Elevating of C1q, C1, C3 activity was the most dramatic. Levels of C3, C4 and C1 FA negatively correlated with hemoglobin content ($p<0.001$; 0.002; 0.01 respectively). Also we have revealed positive correlation between C3 FA and degree of amiothrophy. Galactose content in RA patients' IgG varied from 1.02 to 1.48 per one chain (1.78 in donor's IgG) and galactose content decreased along with elevating of RF, CRP, seromucoid and plasma viscosity, i.e. laboratory indexes of disease activity. Galactose deficiency also coincided with enhancing of C3 FA and CH50 ($p<0.02$). In patients with low galactose content in IgG circulating immune complexes (CIC) of lower molecular weight were found (approximately 6 IgG molecules per complex compared with 10-15 molecules in donors). This patients' group had higher Ritchi's index than group with greater CIC ($p<0.05$). Positive correlation between CIC level and duration of morning stiffness was revealed ($p<0.03$). Thus, data obtained suggest the degalactosylation of IgG in RA to be one of the pathogenic steps in RA development and to play important role in disease progression. Elevating of FA of CC may be due to high CIC level and reflects one of the pathways directed on CIC clearance.

S6.10

Stress-Related Changes in Human Serum Glycoproteins

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Various studies conducted during the past years have clearly demonstrated correlation between the exposure to stress and the development and course of many human diseases, from simple virus infections to cancer. On the molecular level this is manifested in numerous complex biochemical changes, from altered activity of enzymes, to novel expression of specific genes. Recent results, including ours, indicate that glycosylation, as the most important posttranslational modification, also plays an important role in the stress response.

Using a set of digoxigenin-labeled lectins we have analyzed changes in glycosylation patterns in sera of prisoners released from war camps. Among others, two major alterations were detected in nearly all detainees: (i) appearance of a novel 45 kD DSA- and SNA-reactive glycoprotein; and (ii) a significant increase in the amount of 37 kD SNA- reactive glycoprotein. Subsequently we have analyzed some other models of human stress (professional soldiers, drivers involved in motor vehicle accidents, *etc.*) and found very similar changes, including a significant increase in concentration of DSA and RCA reactive 57 kD glycoprotein. It is interesting that even stress caused by prenatal exposure to diagnostic Doppler ultrasound induced specific glycosylation changes, *i.e.* appearance of novel glycoproteins and gangliosides.

By combining classical chromatographic methods and affinity chromatography on immobilized lectins we have established purification procedures for 45 and 57 kD glycoproteins and prepared samples for structural carbohydrate and protein analysis. Preliminary blotting experiments to determine the structure of carbohydrate chains were performed.

S6.11

SERUM GALACTOSYLTRANSFERASE IN RHEUMATOID ARTHRITIS

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Objective- To determine the expression of the glycosylation enzyme β,1-4 galactosyltransferase (GTase) in the sera of patients with rheumatoid arthritis (RA), and assess its correlation with disease activity parameters.

Methods- Serum GTase levels were determined and compared in the sera of 94 patients with RA and 95 healthy controls by an enzymatic based biochemical assay measuring the transfer of ^{3}H-Galactose to an exogenous acceptor molecule. Physicians global estimate of disease activity, synovitis, joint score, C reactive protein (CRP), erythrocyte sedimentation rate (ESR), and haemoglobin concentration (Hb) were evaluated as parameters of disease activity.

Results- GTase activity was significantly higher in the sera of patients with RA compared with the control group ($p<0.0001$) and its increase paralleled the increase in disease activity. Despite the wide inter-individual variation serum GTase was significantly higher in the active ($n=53$) compared with the inactive group ($n=26$; $p<0.05$) and showed a significant positive correlation with CRP ($n=31$; $r=0.787$, $p<0.005$) and ESR ($n=69$; $r=0.310$, $p<0.05$). Serum concentrations of haemoglobin correlated inversely with GTase activity, but this did not reach significance.

Conclusions- GTase may be a relevant marker of disease activity in RA. Its increased expression in serum may be an indirect indicator of RA associated IgG hypo-galactosylation, thus supporting the hypothesis that glycosylation has a role in the pathogenesis of RA.

S6.12

β1,4-Galactosyltransferase Isoenzyme Changes In Serum Of Patients With Rheumatoid Arthritis

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Objective: To investigate possible specific β1,4-GTase isoenzyme changes in serum of patients with rheumatoid arthritis (RA).

Design & Methods: β1,4-galactosyltransferase (GTase) is a soluble and membrane bound enzyme which is responsible for the transfer of galactose from UDP-galactose to terminal N-acetylglucosamine (GlcNAc) residues of complex asparagine-linked oligosaccharides. β1,4-GTase has been shown to be reduced in B and T lymphocytes in patients with RA and is associated with the hypogalactosylation of IgG in this disease. Using solution phase isoelectric focusing, we have determined the isoenzyme profiles of GTase in healthy individuals (HI) (n=9), and patients with psoriatic arthritis (PsA) (n=9) and RA (n=8). GTase activity was determined using a previously reported assay, in which ^{3}H-galactose is transferred to ovalbumin.

Results: Comparison of GTase activity profiles demonstrated that the RA group was significantly different from both the PsA patients and HI ($p < 0.01$). In 7 patients with RA, 8 HI and 6 PsA, two fractions showed distinct peaks of activity. The first peak of activity formed at pH 4.5 (range 4.3-4.7), pH 4.6 (range 4.4-5.0) and 4.65 (range 4.35-5.0), whilst the second peak of activity focused at pH 5.0 (range 5.0-5.1), pH 5.2 (range 5.0-5.4) and 5.20 (range 5.0-5.50) in the RA, HI and PsA populations respectively. There was a significant shift in the pI position of the second peak when comparing the RA group to the healthy individuals (p=0.006). A significant increase in GTase activity was noted in the RA second peak (p=0.008) when compared to HI and PsA.

Conclusion: Two main peaks of serum GTase activity have been identified and there is evidence that RA associated serum GTase isoenzymes occur. The RA GTase isoenzyme profile is significantly different from the HI and PsA as there is acidic skewing of the first peak, a prominent second peak concentrated over a narrow pH band (pH 4.97-5.11) and a loss of activity in the pH range 5.20-5.60. The significance of these findings now needs to be determined with reference to RA pathogenesis and the enzyme contained within these peaks fully characterised.

S6.13

Glycosylation profiles of IgG paraproteins in multiple myeloma (MM)

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Multiple myeloma is a diffuse neoplasm of bone marrow plasma cells associated with the presence of a paraprotein in blood and or urine. Chemotherapy induces a period of stable disease (plateau phase), not requiring therapy, in two thirds of cases. The plateau phase persists with a half life of ~18 months with disease ultimately reactivating and necessitating further chemotherapy. The median survival time is ~3yrs.

A longitudinal study has been made of the glycoform profiles of polyclonal IgG1,2&4 and the monoclonal IgG3 component in three patients with MM. The glycoform profile of the polyclonal IgG was shown to be stable, however, there was an increased G0 component, relative to age matched controls. The glycoform profile of the IgG paraproteins differed from each other and varied over the course of disease. We speculate that these changes may be indicative of changes in plasma cell microenvironments or the emergence of subclones. Altered IgG glycosylation within subclones of the neoplastic cells would suggest that other glycoprotein products could similarly have altered glycosylation states and hence a different patho-physiology.

An apparent preference for galactosylation on the α1-3 arm relative to the α1-6 arm for a majority of IgG3 paraproteins was observed. Further study revealed a correlation with heavy chain allotype. Thus, for seven IgG3.G3m(b) proteins the preference is for α1-3 galactosylation whilst for four IgG3.G3m(g) proteins the preference was α1-6. Previously we showed that for IgG2 paraproteins there is an apparent α1-3 preference. These findings demonstrate that fine differences in primary structure can influence IgG as a substrate for β1-4 GTase.

S6.14

Sugar Printing Rheumatic Diseases: A Potential Method for Diagnosis and Differentiation using Immunoglobulin G Oligosaccharides

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Introduction: Immunoglobulin G contains on average 2.5 nitrogen linked di-antennary (1,3 and 1,6 linked) oligosaccharide molecules which have heterogeneity at the non-reducing terminal. The Fc region is always glycosylated and there is variable glycosylation at the Fab portion. Rheumatic diseases are associated with changes in their immunoglobulin G oligosaccharide profile. For example, rheumatoid arthritis is associated with hypogalactosylation. Reduced galactose at the Fc region causes rheumatoid factor to bind more strongly and this is associated with immune complex formation. Additionally, mannose binding protein will bind to the exposed N-acetylglucosamine structures and this results in complement activation. To determine whether specific glycosylation profiles and hence pathogenic mechanisms are associated with rheumatic disease, detailed IgG oligosaccharide profiles were obtained from a spectrum of conditions.

Methods: HPLC analysis was carried out to identify a total of 16 distinct IgG derived oligosaccharide structures from patients with rheumatoid arthritis (n=5), SLE (n=10), primary Sjögren's syndrome (n=6), ankylosing spondylitis (n=10), juvenile chronic arthritis (n=13), psoriatic arthritis (n=9) and healthy individuals (n=19).

Results: The proportions of the different oligosaccharide structures in each disease group was significantly different each other and the healthy controls ($p < 0.01$). In particular, specific associations were found with each disease group with respect to the proportion of galactose (No. = g0, g1, g2), N-acetylglucosamine (b=bisecting), sialic acid and fucose (f). Oligosaccharide profiles comprising these oligosaccharide changes were disease specific, giving a unique sugar print for each disorder. For example, the RA sugar print showed an increase in g0, g0f, g0fb and reduction in g1(1,3)b, g1(1,6)f, g2f structures. In contrast the SLE sugar print showed an increase in g1(1,6), g2, g2fb and reduction in g1(1,3)b, g1(1,6)f and g2f structures.

Conclusion: Disease specific IgG sugar prints have been demonstrated. This suggests that disease specific glycosylation of IgG occurs during synthesis and may indicate that IgG is involved in different pathogenic mechanisms in each disease. It also raises the possibility that sugar prints may be useful in the diagnosis and differentiation of rheumatic diseases.

SESSION 7: GLYCOSYLATION AND DISEASE (PART II)
CHAIRPERSONS: AJIT VARKI & CLAUDINE KIEDA

S7.1

Oligosaccharide profiling of acute-phase proteins: a possible strategy towards better disease markers G. A.Turner and M.T.Goodarzi, Department of Clinical Biochemistry, The Medical School, Newcastle upon Tyne, UK

The large variety of carbohydrate structures on glycoproteins and the ability of these structures to change in response to the environment provide us with an opportunity to develop better disease markers. Using monosaccharide analysis, we have previously shown that the carbohydrate composition of the acute-phase proteins, alpha-1-proteinase inhibitor (API) and haptoglobin (Hp), can vary in disease. Changes in fucosylation were a common finding against a background of changes in sialylation and branching. However, this approach was limited because it was difficult to determine the precise structural changes from the data or to develop the methodology as a diagnostic technique. In order to make further progress we developed a new procedure to remove the oligosaccharides completely and reproducibly from a very small amount (50μg) of purified serum glycoprotein using PNGase F and to analyse them on the Dionex chromatography system. The sialylated oligosaccharide composition of API and Hp was investigated in 10 healthy individuals and 49 patients with different diseases (rheumatoid arthritis, Crohn's disease, breast, stomach and ovarian cancer). Using this technique 26 peaks could be routinely and reliably detected. Unique oligosaccharide profiles were obtained for both proteins in all diseases, except in breast and ovarian cancer, where the Hp profiles were very similar. Particularly striking were the changes in the profile of Hp oligosaccharides in Crohn's disease and stomach cancer. Some of the changes involved peaks that could be easily identified (monosialylated diantennary, bisialylated diantennary, fucosylated bisialylated diantennary, trisialylated triantennary). A semi-quantative lectin-binding assay was developed to help to identify some of the unknown peaks. As more oligosaccharide peaks are identified it should be possible to use this information to develop automated technologies that can detect disease-specific carbohydrate profiles.

S7.2

Alterations in the Unique M Glycan Prevent Hepatitis B Virus Formation – A Potential Therapeutic Target.

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The envelope of human hepatitis B virus contains three distinct envelope glycoproteins termed L, M, and S. All three glycoproteins contain a single common N-linked glycosylation site near their carboxyl terminal end while M contains an additional unique N-linked site near it's amino terminus. An HBV infected cell secretes both an infectious viral particle as well as a non-infectious sub-viral particle. Inhibition of N-linked glycosylation with tunicamycin, or the glycosylation pathway with N-butyl-deoxynojirimycin (NB-DNJ), leads to an arrest of viral but not sub-viral particle secretion. Glucosidase I inhibition with NB-DNJ leads to the secretion of sub-viral particles that lack the M protein but still contain complex glycan structures. Further experiments determined that the M protein was hyperglucosylated and localized to lysosomal compartments. HBV expression vectors which lacked either all the glycan sites, or the unique M glycan site, were unable to produce virus. However, alterations within the copmmon S glycan site had no effect on virion production. These results identify the unique M glycan site as playing a crucial role in the production of HBV and highlights its role as a potential therapeutic target.

S7.3

Escape from ER Quality Control: In Glucosidase Inhibited Cells, HBV Glycoproteins Become Dysfunctional, Aggregate and have Long Half Lives, but still leave the ER

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Since the role of glycosylation trimming in glycoprotein fate and function is unclear and hepatitis B virus (HBV) glycoproteins are essential to the virus life cycle, the behavior of HBV glycoproteins in cells in which endoplasmic reticular (ER) glucosidase has been inhibited was evaluated. ER glucosidases process N glycan. HBV is not secreted from cells in which glucosidase has been inhibited. Viral DNA accumulating within the glucosidase inhibited cells is most likely to be located within lysosomal compartments, accompanied by capsid and envelope proteins. Pulse chase experiments show that although the viral glycoproteins (L, M and S) are not efficiently secreted and are dysfunctional, the retained glycoproteins were not rapidly degraded and exited the ER, appearing as aggregates with intracellular half lives exceeding 20 hours. Thus, surprisingly, the defective glycoproteins, escaped ER "quality" control. Since they were, however, retained within the cell, it appears that a level of quality control occurred at a post ER step. Moreover, by 24 hours after synthesis, a substantial fraction of the detained glycoproteins appeared to return to the ER although a considerable amount was also found in the lysosomes. This is thus the first detailed tracking of HBV glycoproteins throughout the cell as well as evidence of aberrant ER departure, protein aggregation and half life as a consequence of inhibiting glycosylation processing. It is hypothesized that failure to process N-glycan causes HBV glycoproteins to aggregate and that impaired protein-protein interactions and trafficking are the result of misfolding. The possibility of using inhibitors of glycosylation processing and "dominant negative" defective HBV glycoproteins as an antiviral therapy for HBV infection will also be discussed.

S7.4

Inducible expression of sialyl Lewis^x and sialyl Lewis^a, essential components of selectin ligands, in breast cancer. Comparison of epithelial and endothelial expression in primary versus metastatic lesions.

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Tumor cells can invade and generate metastasis either via lymphatics or blood vessels. When in blood circulation the tumors cells have to adhere to the vessel wall lined by endothelium before they can extravasate. Several families of adhesion molecules have been identified to play a role in the extravasation cascade. Due to the initiating role of selectins and their sialyl Lewis^x (sLe^x) and sialyl Lewis^a (sLe^a) containing ligands in this cascade we have now analysed their role in the generation of metastatic breast carcinoma lesions. We have analysed the expression of endothelial E- and P-selectin on one hand and epithelial (carcinoma) sLe^x and sLe^a expression on the other hand in normal tissues as well as in primary and metastatic breast carcinoma lesions within individual patients. We show that the endothelium in metastatic lesions express high levels of both the E- and P-selectin. While the normal breast epithelial cells do not express sLe^x or sLe^a their expression was strogly enhanced in primary breast carcinoma lesions. Furthermore the expression of sLe^x and sLe^a were at an even higher level in most of the patients' (9 out of 12) metastatic compared to primary lesions. This data support the hypothesis that while in blood circulation the sLe^x and/or sLe^a expressing carcinoma cells have a better chance to make contract with endothelium expressing E- and P-selectin, extravasate at these sites and generate new metastases.

S7.5

Altered lectin binding to the O-linked glycans of IgA1 in children with Henoch Schonlein purpura (HSP) restricted to patients with clinical nephritis

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We have previously described abnormal O-glycosylation of serum IgA1 in IgA nephropathy, detected by increased binding of the GalNAc-specific lectin *Vicia villosa* (VV) (1). Henoch-Schonlein purpura is a form of systemic vasculitis associated with IgA deposition in various sites, often but not always, including the kidney. The aim of this study was to investigate whether the O-glycosylation defect of IgA1 seen in IgAN is also found in HSP, and whether HSP patients without renal involvement display the same abnormality.

Serum was obtained from 28 children with HSP with nephritis, 21 children with HSP without nephritis, and 22 age and sex matched controls. Ammonium sulphate precipitates were prepared from the sera, and applied to anti-IgA coated immunoplates. Biotinylated VV lectin was then applied, and binding detected with peroxidase-conjugated avidin and OPD substrate. Results were expressed as OD at 492nm.

Serum IgA from children with HSP and nephritis showed significantly higher VV binding than matched controls (0.534 ± 0.038 vs 0.410 ± 0.010 respectively, $p<0.004$). However, serum IgA from children with HSP but without nephritis had significantly lower VV binding than those with nephritis (0.406 ± 0.019 vs 0.534 ± 0.038 respectively, $p<0.005$), and did not differ from controls.

In conclusion, these data show that serum IgA from patients with HSP and nephritis is abnormally O-glycosylated. This abnormality is similar to that seen in IgAN. However, patients with HSP but no nephritis do not display this abnormality. Altered O-glycosylation of IgA1 may be a marker of disease severity in HSP, or may contribute directly to renal IgA deposition and the development of glomerular damage in HSP.

Reference (1) Allen AC et al Clin Exp Immunol 1995;100(3):406.

S7.6

Identification of oligosaccharides associated with poor outcome in breast cancer.

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Each year 26,000 new breast cancers are detected in the UK. The main cause of death from breast cancer is the metastasis of the primary cancer to distant sites. There is growing evidence that changes in oligosaccharides occur on glycoproteins associated with malignancy and that oligosaccharides may be involved in the metastatic pathway. Most of the work using human tissues to date only provides limited information regarding the oligosaccharide structures involved. Cancer tissues removed from patients are conventionally fixed in formalin and stored embedded in paraffin-wax blocks. These tissues offer a source of primary tumour material with long patient follow-up data.

Hydrazine can be used to release N-linked oligosaccharides from formalin fixed tissues which have been stored in paraffin-wax blocks. We have used this approach to release oligosaccharides from breast tumour tissues excised at the Middlesex and University College Hospital, London between 1978 and 1981. Oligosaccharides are labelled with a fluorescent tag and evaluated using anion exchange chromatography and hydrophillic interaction chromatography. Breast tumour specimens have been matched for age, grade and stage and comprise two groups, no sign of recurrence or dead within 5 years, ten samples per group, pre- and post menopausal.

Initial results indicate that there may be an alteration in the sialylation in oligosaccharides of breast cancers which progress to form metastases in the patient. Further samples are being assessed in this project, mapping oligosaccharides associated with poor outcome in breast cancer. Glycopeptides indicated to be changed from these studies will be used to study immune responses to tumours using patient sera.

S7.7

FUNCTIONAL CHANGE OF SERUM IMMUNOGLOBULIN G IN DIABETIC PATIENTS

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Our previously reported data have documented that human NK cells interact in vitro via FcγRIIIA with monomeric cytophilic IgG (mIgG). This binding induced a dose dependent down-regulation of NK cell activity. On the other hand we have also noted quantitative and qualitative changes of serum IgG in diabetics. To extend these observations, in the present studies we evaluated the modulatory effects of mIgG isolated from a group of 50 diabetics patients on the cytolytic function of human NK cells isolated from normal, healthy donors. In comparison with the percentage of inhibition induced by normal mIgG (66.8±22.9) (n=15) significat ($p<0.05$) reduction of such inhibition was found with mIgG separated from serum of patients with either type 2 (49.6±25.6) (n=18), or with short duration of disease (<1year) (43.8±30.9) (n=11). A significant decreased capacity to inhibit NK activity was, also observed with IgG separated from the sera of 60% of patients with high level of HGA1 (>10%). We did not observed significant alterations of the inhibitory effect of mIgG obtained from patients with either type 1 diabets or with a long duration of disease. Our results suggest:1) the nonenzymatically glycosylated serum IgG in diabets apparently affects its property to modify NK cell activity; 2) clinical characteristics of diabets are involved in this process

S7.8

Heterogeneity of Cell Wall Glycolipids Paralleled with Virulence in *Mycobacterium tuberculosis* and *M.avium* Human Isolates and Serotypes

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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 fatty acids or carbohydrate moiety. As the result, 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. In cases of *M.avium*, serotype specific peptidoglycolipids showed characteristics as virulent factor. The results show that existence of these toxic glycolipids profoundly contributes to the virulence of *M.tuberculosis* and *M.avium*. The combined effect and role of each glycolipid will be discussed.

S7.9

Effect of pregnancy on glycosylation of α1-acid glycoprotein in rheumatoid arthritis. *Ellen C. Havenaar[1],John Axford, Els C.M. Brinkman-van der Linden[1], Azita Alavi[2], T. Spector and Willem van Dijk[1], [1] Dept. Medical Chemistry, Vrije Universiteit, 1081 BT Amsterdam, The Netherlands.* [2]Academic rheumatology unit, Dept. Cellular and Molecular Sciences, St. George's Hospital Medical - School, London SW17 0RE, UK, [3] St Thomas Hospital, London, UK

In RA, changes in glycosylation of the acute phase protein α1-acid glycoprotein (AGP) occur and increased fucosylation of AGP has been shown to correlate with RA disease activity. During pregnancy in healthy individuals, the glycosylation of acute phase proteins is subject to marked changes. The aim of this study was to determine whether changes in glycosylation of AGP occurred in a longitudinal study of pregnant RA-patients, and were related with disease activity. Patients were divided into 3 clinical categories: remission (n=8), relapse (n=5) and unchanged (n=7). The extent of branching and fucosylation of AGP was determined in total serum by crossed affino immunoelectrophoresis with concanavalin A and *Aleuria aurantia* lectin, respectively, as affinity components in the first dimension gel, and a precipitating monospecific antiserum directed towards AGP in the second dimension gel.The degree of branching of the glycans of AGP was found to be increased 2-fold from week 5 to 35 in normal pregnancy, and 1.2- to 1.5-fold in RA-pregnancy during the same period. Furthermore, in normal pregnancy, remission and relapse, the amount of fucosylated AGP was decreased relative to the fucosylation after birth. In the unchanged group, however, the fucosylation decreased from 160% in week 5 to 100% in 10 relative to values after birth, and remained constant thereafter. It can be concluded that pregnancy induces changes in glycosylation of AGP both in healthy individuals and in patients with RA. The magnitude of changes can be classified as follows: healthy>remission+relapse>unchanged.

S7.10

THE GLYCOSYLATION PATTERN OF SOLUBLE IgG SUBCLASSES IN THE MONITORING OF PATIENTS WITH RHEUMATOID ARTHRITIS

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As an attempt to relate clinical activity with oligosaccharide content of soluble and complexed IgG subclasses, 30 patients with active (group A) rheumatoid arthritis (RA), 30 with the inactive form of the disease (group B) and 30 normal controls (group C) were investigated. Following PEG-precipitation of immune complexes, the sugar compositions of the soluble and complexed IgG subclasses were estimated, using a panel of ELISA developed in our laboratory. These tests are based upon the use of deglycosylated anti-IgG subclass monoclonals as capture agents, and sugar-specific biotinylated lectins combined with a second layer of HRP-conjugated streptavidin as a revealing agent [*Sambucus nigra* binds to *N*-acetyl neuraminic acid (NANA), *Ricinus communis* agglutinin to galactose (Gal) exposed by neuraminidase, and *Bandeiraea simplicifolia* II to *N*-acetyl-glucosamine (GlcNAc) exposed by neuraminidase plus β galactosidase].

With regard to NANA, differences between the three groups of individuals were significant : $p<0.005$ for soluble IgG_2 and IgG_4 between group A and group B, for IgG_1 and IgG_3 between group A and group C, and for IgG_2, IgG_3 and IgG_4 between group B and group C. Significant differences of Gal and GlcNAc ($p<0.0001$) appeared for all subclasses of soluble IgG between group A and group B, as well as between group B and group C. The rank order of Gal was group A<group C<group B, whereas that of GlcNAc was group C<group A <group B. In contrast, the amounts of NANA, Gal and GlcNAc were similar in the complexed IgG subclasses from groups A, B and C.

To conclude, not only were the glycosylation abnormalities of the IgG subclasses confirmed in RA patients, but these patterns were proven to be related to disease activity. Serial evaluation of oligosaccharides in soluble IgG subclasses might thus be useful in the monitoring of RA patients.

S7.11

Relationship between cell surface glycosylation and immunogenicity of rat colon carcinoma cells

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Using a rat model of colon carcinoma, we previously observed a relationship between the level of expression of H blood group antigens (Fuc1-2Gal structures) and the tumorigenicity of cancer clones. In this experimental model, the tumorigenicity is largely dependant on the ability of the clones to escape the imune system. We also observed that a combination of interleukin-2 and sodium butyrate (NaB) could cure rats from peritoneal carcinomatosis, while either agent alone was ineffective. It appeared that NaB treatment increases the cells immunogenicity in a vaccination assay. NaB treatment also modifies cell surface glycosylation. On a tumorigenic clone (PRO), after NaB treatment, reactivity with antibodies against H types 1 and/or 3 was lost, while binding sites for the Maackiá amurensis lectin (MAA) appeared (NeuAc2-3Gal). To delineate the importance of these terminal glycosylations, in determining the cells behavior, we first used a spontaneously regressive clone (REG) which is H negative and MAA positive. After transfection with the H fucosyltransferase, the cells became H positive and their MAA reactivity dropped. These transfectants acquired the ability to grow in syngeneic immunocompetent animals. We then used the highly tumorigenic clone PRO which is spontaneously H positive and MAA negative. These cells were transfected with the cDNA coding for the sialyltransferase ST3Gal I. The transfectants acquired MAA reactivity, lost H types 1 and/or 3 reactivity, like NaB treated parental cells. These transfectants were less tumorigenic in syngeneic rats, some animals being able to completely reject their tumor. Thus, in this experimental model, an α2-3sialyltransferase and an α1-2fucosyltransferase can compete for the same acceptor substrates. The presence of NeuAc2-3Gal structures correlative with the lack of Fuc1-2Gal structures, as obtained after NaB treatment of tumorigenic cells, is responsible for a decreased tumorigenic potential of the cells in fully immunocompetent animals.

S7.12

Breach of the Blood Brain Barrier Occurs via Capillary Endothelium During Acute Herpes Simplex Encephalitis

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Herpes Simplex Encephalitis (HSE) is a rare manifestation of Herpes Simplex Virus (HSV) infection, but is the commonest cause of fatal encephalitis is immunocompetent individuals.

While the intrathecal immune response to HSV is understood in general, detailed aspects of the infection remain poorly understood. We have begun an analysis of the molecular immunopathogenesis of HSE using archival brain tissue from fatal cases of the disease.

As in many other encephalitides perivenular lymphocyte cuffs are conspicuous in HSE and it is generally assumed that these are the source of lymphocytes entering the inflamed brain.

We have examined infected brain tissue by *in situ* hybridisation, PCR and immunocytochemistry to locate virus and by histochemical , lectin and monoclonal antibody histochemistry to detect antigens and glycans indicative of cellular reaction and movement.

During acute HSE capillary endothelium in virus-rich areas showed altered glycosylation and up-regulation of Sialyl Lewis^X antigen (SLe^X). Lymphocytes were adherent to the endothelium and numerous in the neuropil near capillaries. Some glial cells in the same area also expressed SLe^X strongly. There was no SLe^X expression on venular endothelium but other venular glycans changed. Cuffs formed near virus-infected areas but persisted after virus disappeared. They contained lymphocytes and macrophage-like cells within a tenuous perivascular sheath, but there was no evidence of lymphocytic migration into the adjacent neuropil.

Capillary and venular responses in HSE are, therefore, functionally distinct, with lymphocytes contributing in different ways to each.

S7.13

Differential Serodiagnosis of Tuberculosis or *M.avium* Infection with Cord Factor (trehalose 6, 6'-dimycolate) and Glycopeptidolipid Antigens

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Mycobacterium avium complex (MAC) is one of the most important pathogen coinfected with HIV (AIDS) and a typical intracellular parasite as well as *M.tuberculosis*. It is also focused that *M.avium* infection causes immunosuppression especially in the cellular immunity of host animals, and specific serotype-subspecies such as serotype-2, -4 or -8 can be isolated frequently in human infection in AIDS. Previously, we have reported that the detection of anti-cord factor antibody in active and inactive tuberculosis patients is useful for early clinical diagnosis of tuberculosis. For the differential diagnosis of tuberculosis and non-tuberculous mycobacteriosis, we have examined whether ELISA test using cell surface glycolipid antigens, cord factor (TDM) and glycopeptidolipid (GPL), is applicable or not. Anti TDM IgG antibodies of 40 patients sera infected with *M.avium* (MAC) were highly reactive against *M.avium* cord factor (MA TDM), while lowly reactive against *M.tuberculosis* cord factor (MT TDM). About 60 percent of the patient sera excreting MAC showed seropositive against MA TDM and seronegative against MT TDM. About 30 percent showed seropositive against both MA TDM and MT TDM. Among the patient sera showing the high titer of antibody against MA TDM, but low against MT TDM, several cases showed highly reactive against *M.avium* serotype 4 GPL antigen. The results showed the combined use of TDM (MA TDM and MT TDM) and GPL antigen for ELISA with patient sera is useful for the precise diagnosis of *M.avium* or *M.tuberculosis* infection.

S7.14

N-acetylgalactosamine containing glycoconjugates and the invasive and metastatic capacity of melanoma cell lines.

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Oligosaccharides appear to play similar roles in metastasis and inflammation. Our group has previously established a nude mouse model in which two different melanoma lines (FEMX and LOX) show very different capacity to establish pulmonary metastasis. These previous studies showed that the LOX cells, which have highly increased expression of HPA-binding glycoconjugates compared to the FEMX- cells, was associated specifically with pulmonary metastasis. In this work we have used this melanoma model to further examine the possible role of these glycoconjugates during melanoma metastasis. In vivo experiments: Nude mice were given $1x10^5$ ^{125}I-labelled cells (FEMX or LOX) by intravenous tail vein injection. At different time intervals after injection the animals were killed and the lungs were removed and cell bound reactivity was assessed in a gamma counter. During the first 12 hours, equal fractions of LOX and FEMX were retained in the lung tissue. In vitro experiments: Adhesion to HUVEC cells was tested in a static assay at 37°C. Invasive potential was tested using a Matrigel coated filter. Various N-acetylgalactosamine specific probes were used as blocking reagents. No differences between LOX and FEMX was seen in the adhesion to stimulated HUVECs, and no inhibition of adhesion to endothelium was observed after blocking selected HPA-binding glycoconjugates. LOX was significantly more invasive in the Matrigel assay than FEMX, and preliminary results indicate that invasion of LOX, but not FEMX, can be inhibited by HPA-lectin and a specific N-acetylgalactosamine containing probe.

In conclusion: Our results suggest that N-acetylgalactosamine containing glycoconjugates may be important for melanoma cell-matrix interactions involved in pulmonary specific metastasis.

S7.15

Functional activity (FA) of complement components (CC) and degalactosylated IgG in rheumatoid arthritis (RA) patients.

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Deficiency of IgG galactosylation in RA patients is widely described, but consequences of this phenomenon are under investigation.

We have studied 12 RA patients with confirmed diagnosis (ARA criteria, 1988), all women from 28 to 64 years old with high disease activity. 42 healthy age-matched women were in control group. Galactose content in IgG samples was determined by HPGLC, FA of CC (C1q, C1-C5, CH50) - by microhemolytical assay, other test were made by routine techniques.

We have found all RA patients had significant increase of FA of CC

and C3 and C4 levels compared with donors ($p<0.001$). Elevating of C1q, C1, C3 activity was the most dramatic. Levels of C3, C4 and C1 FA negatively correlated with hemoglobin content ($p<0.001$; 0.002; 0.01 respectively). Also we have revealed positive correlation between C3 FA and degree of amiothrophy. Galactose content in RA patients' IgG varied from 1.02 to 1.48 per one chain (1.78 in donor's IgG) and galactose content decreased along with elevating of RF, CRP, seromucoid and plasma viscosity, i.e. laboratory indexes of disease activity. Galactose deficiency also coincided with enhancing of C3 FA and CH50 ($p<0.02$). In patients with low galactose content in IgG circulating immune complexes (CIC) of lower molecular weight were found (approximately 6 IgG molecules per complex compared with 10-15 molecules in donors). This patients' group had higher Ritchi's index than group with greater CIC ($p<0.05$). Positive correlation between CIC level and duration of morning stiffness was revealed ($p<0.03$).

Thus, data obtained suggest the degalactosylation of IgG in RA to be one of the pathogenic steps in RA development and to play important role in disease progression. Elevating of FA of CC may be due to high CIC level and reflects one of the pathways directed on CIC clearance.

SESSION 8: GLYCOTHERAPEUTICS (PART I)
CHAIRPERSONS: ROY JEFFERIS & RICHARD CUMMINGS

S8.2

Bacterial lipopolysaccharides: candidate vaccines to prevent *Neisseria meningitidis* and *Haemophilus influenzae*

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Haemophilus influenzae (Hi) and *Neisseria meningitidis* (Nm) are bacterial commensals of the upper respiratory tract of humans which also have pathogenic potential. These organisms cause serious diseases including septicaemia and meningitis. Major virulence factors and potential targets for protective immune responses include two major surface carbohydrate antigens: capsular polysaccharides and lipopolysaccharides (LPS) respectively. The structures and antigenic properties of LPS are of importance in the development of candidate vaccines against: i) Hi which lack the type b capsular polysaccharide and ii) Group B strains of Nm.

LPS molecules are the major antigens of the outer leaflet of the cell envelope of all gram-negative bacteria. In general, lipid A is highly conserved across the many genera of gram-negative organisms, the inner core is conserved for a particular genus and the outer core is variable within a species. Our approach to developing vaccine candidates in Hi and Nm involves defining the inner core structures through a combination of molecular genetics and high resolution structural analyses (NMR and MS). The aim is to identify epitopes of the inner core which are accessible to host immune responses and which can be used as immunogens to elicit protective immunity. The availability of the whole genome sequence of Hi has greatly facilitated the identification of the large number of genes involved in the biosynthesis of the core oligosaccharides. The LPS derived from a series of mutants at successive stages in the biosynthetic pathway have been used for structural analyses. Using computer assisted modelling, this has allowed us to generate a space filling model of the core oligosaccharides which provide a starting point for the selection of candidate epitopes which will be further tested for their potential utility as vaccines.

S8.3

Development of Retroviral Vectors for Transfer of Glycosyltransferase Genes

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Insertion or removal of glycosyltransferase genes may result in the alteration of phenotype and biological properties of the target cell. Although gene transfer technology has developed significantly there are still problems with transfer efficacy and specificity, long term and level of gene expression. Due to the feasibility of stable transduction of dividing cells retroviral vectors (RV) are widely used. They employ 5'LTR to promote expression of therapeutic gene and anabiotic resistance genes driven by separate internal promoters. Such constructs however, yield resistant cells of which only 5–10% express the therapeutic gene. To overcome this phenomenon a dicistronic gene was created in which an IRES sequence was used to join therapeutic and selectable gene cDNAs. LacZ as a marker and Neo as a resistant gene were used. Cassette LacZ-IRES-Neo was inserted into transcriptional region of three various RV. Moreover double-copy dicistronic vector based on MSCV was constructed by insertion of a cassette containing LacZ-IRES-Neo driven by exogenous HCMV-IE promoter into U3 region of 3'LTR. Constructed RV were inserted into PA-317 packaging cells. Recombinant viruses were tested in several human and murine cell lines. Since double-copy dicistronic vectors provided highest and long lasting expression of marker gene in every selected cell it has been chosen for further studies. Accordingly several glycosyltransferase cDNAs including human 1,4βGalTn, 2,6STn, 2,3GalNacST, 2,3GlcNacSTn, FucTnIII,IV,V and VI and murine 1,4GalT short and long cDNAs were inserted into the vector. Finally human 1,4βGalTn cDNA was transduced into murine melanoma cell line B-78-H1. GalTn-B-78-H1 and control cells

(5×10^5) were injected subcutaneously into C57B16 × C3H mice and tumor growth as well as survival were monitored. In control animals formation of tumors was observed after three weeks, while in mice injected with GalTn-B-78-H1 cells after five weeks. However, survival time did not differ significantly between two groups studied.

S8.4

Crystalline Bacterial Cell Surface Layers (S-Layers) as Carriers for *Streptococcus pneumoniae* Vaccines

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The use of crystalline bacterial surface layer proteins as combined vaccine carrier/adjuvants has been explored over recent years. Immunogenic conjugates have been prepared of S-layers with the major allergen of birch pollen, with tumor-associated oligosaccharides, and with oligosaccharide fragments derived from serotype 8 of *Streptococcus pneumoniae*. These prototype serotype 8 oligosaccharide/S-layer conjugates elicited protective antibody responses in animals.

Presently, specialized composite vaccines are being designed for high-risk target groups such as infants or immune-compromised patients. We have developed coupling technology to give good reproducibility with the carbohydrate to protein ratio of the respective S-layer conjugates. The oligosaccharide size appears to be critical for the immunogenicity. To improve their efficacy these prototype vaccines will contain S-layer-oligosaccharide conjugates admixed with purified capsular polysaccharides.

From our results we conclude that S-layers appear to possess good potential as a unique carrier/adjuvant system for conjugate vaccines.

S8.5

GLYCOPROTEIN ALTERATIONS IN RETINAL MICROVASCULAR CELLS BY GLYCATED PRODUCTS.

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The glycocalix is known to determine the relations between the cells of the vascular wall itself or between the endothelium and the circulating blood cells. These specific cell-to-cell interactions are altered in the diabetic retinopathy (loss of pericytes, proliferation of endothelial cells). A mechanism evocated for the development of this complication is glycation. Advanced glycosylation end products (AGEs) are known to accumulate on vascular basal membranes and to affect in vitro the retinal cells proliferation. Since little is known concerning the diabetic environment and the structure or biosynthesis of glycoconjugates, we studied the effects of AGEs on the glycoprotein profiles of retinal cells.
Pericytes and endothelial cells (BREC) from bovine retinal microvessels were cultured in the presence of glycated albumin (AGE-BSA). The cell homogenates were analysed by lectin affino-blotting.
The total protein content is not affected by the glycated products. However, the analysis of the glycoprotein profiles shows specific modifications of the glycosylation pattern for the BREC cultured with AGE-BSA. A significant decrease of terminal glycosylations, α(2,3) sialylation, β–galactosylation and fucosylation, is observed mainly for a 210 Kda glycoprotein, localised in a membrane-enriched fraction. This phenomenom seems to be AGE dose-dependante. This glycoprotein is less modified in pericytes.
These results show that AGEs can affect the biology of diabetic microvascular cells and modify the terminal sugars in the endothelial cells. The enzymatic glycosylation modifications might be implicated in some aspects of the etiopathology of retinopathy, since it is known that altered glycoforms can exhibit different biological properties and affect cellular interactions.

S8.6

A STUDY OF BIOLOGICAL IMPLICATIONS OF THE *Escherichia coli* CAPSULAR POLYSACCHARIDE K1 (CpsK1) O-ACETYLATION FORM VARIATION IN A NEONATAL MOUSE MODEL OF INFECTION.

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E. coli carrying the CpsK1 is a prevalent cause of gram negative neonatal bacteraemia and meningitis in humans, this capsular polysaccharide being identified as an essential virulence factor. The CpsK1, a homopolymer of α(2-8)-linked N-acetyl neuraminic acid, shows form variation resulting from O-acetylation. Each strain has a prevalent expression of the CpsK1 in O-acetylated (CpsK1+) or the non-O-acetylated (CpsK1-) form, showing a relatively high reversion to the opposite phenotype. The biological and pathogenetic implications of this form variation are poorly understood. The CpsK1+ is more inmunogenic that the CpsK1- and therefore, has been pressumed to be less virulent. In an attempt to address this question we have developed a monoclonal antibody (MAb) panel against the Cps of *Neisseria meningitidis* serogroup B, immunochemically identical to the CpsK1-. Two IgM MAbs (MGB12 and MGB15), representative of the two major patterns of crossreactivity with the CpsK1 forms were selected. MGB12 recognizes both CpK1+ and CpsK1- forms, whereas MGB15 shows high specificity for CpsK1-. Both MAbs were used in a neonatal mouse model of infection with *E. coli* K1 strain (O7:K1:-) with prevailing expression of CpsK1- (95% of colonies), and in the control of CpsK1 form variation during bacterial invasion by colony dot-blot assay. In the absence of MAb treatment, form variation selection was not observed during the invasion. The strain maintains an invariable proportion of CpsK1- expression in samples collected from the peritoneal cavity, blood and cerebrospinal fluid. MGB12 affords an high level of protection and no bacteraemic mice were observed after treatment. On the contrary, MGB15 clears only *E. coli* CpsK1-, a few animals remain bacteraemic for CpsK1+ organisms. The infection progresses in a similar way and with a similar LD_{50} (~30 CFU), considering that only CpsK1+ bacteria are in the inoculum of the original unselected strain. The results suggest that whereas form variation does not reinforce virulence *per se*, it could be used by *E. coli* to avoid of immune defense and this stress the importance of the MAb fine specificity selection used in eventual immunotherapeutic approaches to *E. coli* K1 neonatal meningitis.

SESSION 9: GLYCOTHERAPEUTICS (PART II)
CHAIRPERSONS: RAYMOND DWEK & ERIC BERGER

S9.2

The Group B Streptococcal Capsular Carbohydrate: Immune Response and Molecular Mimicry

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The group B streptococcus (GBS) is the commonest cause of lethal infection in the new-born infant. GBS possesses a sialylated carbohydrate capsule which is known to be a major virulence factor. Antibodies to GBS capsule have been shown to be protective but the majority of the population do not have these antibodies. For example, 90% of the population lack antibodies to the type III capsule of GBS (GBS III). Furthermore, 60% of seronegative individuals do not respond to experimental vaccination with GBS III. The basis of the poor immunologic response to GBS III will be examined and the possibility of producing a peptide mimic of the carbohydrate will be addressed.

An in vitro method of stimulating human B cells derived from peripheral blood with GBS III was developed. It is possible to detect anti-GBS III antibody producing B lymphocytes from both seropositive and seronegative donors. The response was shown to be T lymphocyte dependent, with no response in the absence of T lymphocytes nor in the presence of >40% T lymphocytes. Cells from 5 of 6 seropositive donors and 3 of 7 seronegative donors produced specific IgM antibody after culture with antigen. When allogeneic T cells are used in the culture system then a response may also be observed, apparently dependent upon the T cell donor. In addition, when CD8 depleted autologous T cells are employed the response is greater than for the entire T cell fraction. Whereas as the response diminishes with an increasing proportion of T cells it increases as the percentage of CD8 depleted T cells is raised. These results suggest that the control of the human antibody response to the group B streptococcal type III polysaccharide is influenced by T cells and CD8+ cells have a suppressive role in the B lymphocyte response to group B streptococcal type III carbohydrate and T cell control may be responsible for non-responsiveness.

We have performed a single round of panning of a fUSE3 random hexapeptide display library in order to establish whether it will be possible to generate peptides which will bind to anti-GBS type III capsule antibody. To this end, amplified fUSE3 phage library was added to a microtitre well coated with affinity purified anti-GBS III IgG2. After extensive washing, phage was eluted with 1mg/ml of purified GBS III polysaccharide and the number of virions in the eluate were enumerated. Approximately 400 phage particles per 100 µl were eluted from the test well compared to 300 particles per 100 µl from a control (non-coated) well. A microtitre tray was then coated with picked phages and probed with affinity purified anti-GBS III IgG2 antibody. The results of this assay show that with a single round of panning, one clone has the ability to bind to anti-GBS III IgG antibodies and appears to mimic an epitope of GBS III.

S9.3

CELL ADHESION INHIBITORS AS GLYCOTHERAPEUTICS.

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Carbohydrates are increasingly recognized for their information content in biological recognition phenomena, including the binding of antibodies, toxins and microbes to target cells, and the cell-to-cell recognition and adhesion of mammalian cells. With this knowledge have come opportunities for development of carbohydrate-based therapeutics that block the action of carbohydrate binding proteins which mediate processes resulting in pathogenesis. The principal barrier to exploitation of complex carbohydrate-based drugs has been the difficulty in synthesizing this class of compounds economically. Rapid progress has been made in recent years to address this problem using enzymatic and combined chemical and enzymatic synthesis methods. Emphasis will be placed on the breadth of therapeutic opportunities that are available, the scope of synthetic capabilities that can be expected in the future, and what is possible today.

S9.4

Inhibition of HIV-infectivity by human salivary components

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Several studies have indicated that factors present in human saliva can inhibit HIV infectivity *in vitro*. Inhibitory potential is almost exclusively present in mucous saliva, which brings the initial attention on mucins, but also low molecular weight components may be involved.

Thus the aim of the research is to identify which salivary mucins and/or other glycoproteins are involved in inhibition of HIV infectivity.

A set of human salivas, and salivary components, are tested for inhibitory activity on HIV infectivity using the well documented MT2-cytopathic assay in combination with Reversed Transcriptase (RT) activity and syncitium formation assays. These samples include salivas from the various salivary glands (gl. parotis, gl. submandibularis, gl. sublingualis, and the salivary glands situated in palate) collected from of a number of healthy subjects with known bloodgroup and secretor status. These saliva samples are characterized with respect to the composition by SDS-PAGE followed by immuno-blotting using a panel of specific antisera. Besides the cytological tests, binding of salivary components, separated by SDS-PAGE, to molecules present in the HIV envelope, viz., gp120, gp41 will be discussed.

Results of these experiments yield insight in the variation of inhibitory potency between individuals. These studies give clues about the extremely low efficiency of the oral infection route and could lead to the identification of natural inhibitors of viral infection present in human saliva and, perhaps, in other body fluids.

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S9.5

Mannosamine inhibits glycosylation and growth of *Plasmodium falciparum* in culture.

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Native merozoite surface glycoproteins, MSP-1 and MSP-2, offer protection against malaria invasion and are therefore potential vaccine candidates against malaria. It has been shown that recombinant DNA synthesized proteins lacking sugars offer only partial protection suggesting significance of glycosylation. *Invitro* glycosylation and metabolic labelling using [^{3}H]-glucosamine has shown that glycosylation takes place on the surface as well as in the glycosylphosphatidylinositol-anchor regions of MSP-1 and MSP-2. In the current study *invitro* glycosylation, inhibition of glycosylation, and consequence thereof on the parasitemia were investigated. The malarial parasite, *Plasmodium falciparum*, were labelled with [^{3}H]-glucosamine

in synchronous mode and in continuous culture. The radio labelled glycoproteins were extracted in non-ionic detergent and analyzed by SDS-PAGE. Several proteins were labelled with [^{3}H]-glucosamine including MSP-1 and MSP-2. All metabolically labelled glycoproteins extracted in non-ionic detergent were precipitated by human immune serum. For inhibition studies, infected human erythrocytes were cultured with various doses of mannosamine for 48 hours. Effect of mannosamine on parasite survival and glycosylation were followed by metabolic labelling using radioactive [^{3}H]-glucosamine and monitoring parasitemia. Mannosamine significantly reduced parasite survival and synthesis of glycosylated proteins. Inhibition of parasite growth was closely related to the incorporation of redioactive glucosamine. It appears that mannosamine interferes with the biosynthesis of core glycans of glycosylphosphatidylinositol anchor.