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Abstracts

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International Glycoconjugate Organization Award
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Dr. Naoyuki Taniguchi graduated at the Hokkaido University School of Medicine, and obtained his Ph.D. degree from the University in 1972. After serving as associate professor of the Department of Environmental Science, and then of the Department of Biochemistry, Cancer Institute, Hokkaido University, he was appointed Chairman of the Department of Biochemistry, Osaka University Medical School in 1986.

Although he was mainly involved in the study of redox regulation and metal enzymes at the early stage of his career, his interest in the carbohydrate chains of glycoproteins began, since he was asked to collaborate in elucidating the altered glycosylation of rat liver γ-glutamyltranspeptidase by malignant transformation. After having established his own laboratory in Osaka University, he proceeded to purify β-*N*-acetylglucosaminyltransferase (GnT)-III, which was the key enzyme of the malignant alteration of the glycosylation of rat liver γ-glutamyltranspeptidase. Purification of this enzyme led him eventually to the cloning of the gene encoding it. His group also cloned the gene of

GnT-V, and carried out extensive promoter analysis of these two genes. Dr. Taniguchi was involved in early work on the purification of GnT-IV, which led to the cloning of that gene by M. Takeuchi's group. Important methods were developed during these works, namely, fluorescent enzyme assays and affinity chromatography using substrate analogues as ligands. Taniguchi's group has contributed many seminal papers, illustrating that GnT-III and -V play various roles in the development of cancer, and its spread through the body. The GnT-V gene is unregulated by TGFβ in melanoma cells. Transcription factor, ets-1 plays an important role in the transcription for GnT-V. GnT-III gene transfection of B16 melanoma cells suppressed experimental metastasis in mice. Transfection of the GnT-III gene suppressed the expression of hepatitis B related antigen in HB611 cells. Aberrant glycosylation of E-cadherin is expressed in B16 melanoma cells transfected by the GnT-III gene.

Other work has shown that K562 cells, transfected with the GnT-III gene, specifically form colonies in the spleen, suggesting that a specific lectin, which recognizes the bisecting GlcNAc, may exist in the spleen. This led to the identification of annexin V, a bisecting GlcNAc binding protein in porcine spleen.

Taniguchi has shown that N-glycosylation plays a pivotal role in growth factor signaling. The role of heparin-binding EGF in arteriosclerosis and hepatocarcinogenesis was elucidated. It was found that aberrant glycosylation of Trk A and EGF receptors inhibit the dimerization and signaling of NGF and EGF in neuroblastoma cells and glioma cells, respectively.

They also found that deletion of the N-linked sugar chain at Asn-420 of the EGF receptor by site-directed mutagenesis deprived the receptor of its ability to bind to EGF. Another interesting observation is that the mutated receptor oligomerizes spontaneously, and auto-phosphorylates one of its tyrosine residues in the absence of EGF. These results indicated that several essential roles are played by the carbohydrate chain linked to Asn-420 of the receptor.

He has written many reviews, and has presented an impressive number of invited lectures in many different countries and at international meetings. By the middle of the year 2000, he listed 184 peer-reviewed papers in the field of glycobiology. Based on the impact of his publications in the field of glycobiology, and his potential for continuation of his contributions, the members of the selection committee all agreed that Dr. Taniguchi should be selected as the recipient of the 2001 International Glycoconjugate Award.

International Glycoconjugate Organization Award

AL

Lessons from glycosyltransferase genes involved in the branching of N-glycans

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The structures of glycans can vary between species, cell type and the specific glycoprotein. N-glycan branching in glycoproteins dramatically changes during development, immunity and carcinogenesis. To better understand the biological significance of this branching process, 5 types of glycosyltransferases, including *N*-acetylglucosaminyltransferases (GnTs)-III, -IV, -V, -VI, as well as α1,6-fucosyltransferase, were purified, and their genes were cloned. All these genes play pivotal roles in the branching of N-glycans. Remodeling of cell surface glycoproteins by transfection of these "glyco-genes" demonstrated several novel pathways which are associated with tumor metastasis, disease progression, growth factor signaling and immune responses. In this lecture, the biological functions of N-glycan branching in glycoproteins will be discussed from the following points of view:

1. Information derived from transgenic or knockout mice relative to these glyco-genes.
2. Regulation of experimental metastasis by GnT-III and GnT-V.
3. Implications of GnT-III in growth factor signaling
4. Xenotransplantation using the GnT-III transgenic pig.

These results provide new insights into glycobiology and several approaches via "glycomic" approaches will be required to address detailed functions of glycoproteins in the post-genomic era.

Plenary lectures

PL.1

Biosynthesis, secretion and function of Lipid A in Gram-negative bacteria

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Lipid A, the hydrophobic anchor of lipopolysaccharide, constitutes the outer monolayer of the outer membrane in Gram-negative bacteria. Lipid A is a β1'-6 linked disaccharide of glucosamine, acylated at positions 2, 3, 2' and 3', and phosphorylated at positions 1 and 4'. Lipid A not only is required for bacterial growth but also plays an important role in the pathogenesis of infections. Lipid A is an activator of the innate immune system of animals via the receptor TLR-4 and a potent stimulator of cytokine synthesis in macrophages. Over the past decade, we have discovered nine constitutive enzymes required for lipid A biosynthesis in *E. coli* and other Gram-negatives. In *E. coli*, *Salmonella*, *Pseudomonas*, however, additional regulated covalent modifications of lipid A may occur under certain conditions. Recently, we have discovered several new enzymes that are responsible for lipid A modification, and are needed for resistance to defensins and polymyxin. These activities include a palmitoyl transferase, a lipase, a hydroxylase, and a novel oxidative pathway for conversion of UDP-glucuronic acid to the unusual sugar, 4-amino-4-deoxy-L-arabinose, which appears to be incorporated into nascent lipid A via a bactoprenol phosphate intermediate. The lipid A modification enzymes are induced by activation of the PhoP/PhoQ and/or the PmrA/PmrB two-component regulatory systems. In addition, we have recently isolated a temperature-sensitive mutant (WD2) with an A270T substitution in a trans-membrane region of the ABC transporter MsbA. As shown by $^{32}P_i$ and ^{14}C-acetate labeling, export of lipid A and all other phospholipids to the outer membrane is inhibited by ~90 % in WD2 after 30 min at 44 °C. Transport of newly synthesized proteins is not impaired. The lipid A biosynthesis and secretion pathway is an excellent target for the design of new antibiotics against organisms like *E. coli*, *Salmonella*, and *Pseudomonas*, some of which have become resistant to existing antibiotics.

PL.2

N-Glycan processing and glycoprotein folding

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The lumen of the endoplasmic reticulum (ER) is the subcellular site where proteins entering the secretory pathway are glycosylated and acquire their proper tertiary structures. Proteins that fail to correctly fold are first retained in the endoplasmic reticulum and eventually degraded in the proteasomes. Monoglucosylated oligosaccharides ($Glc_1Man_9GlcNAc_2$) formed by glucosidase I and II (GII)-catalyzed partial deglucosylation of the oligosaccharides transferred to Asn residues in nascent polypeptide chains mediate glycoprotein recognition by calnexin (CNX), a membrane-bound ER-resident lectin or by calreticulin (CRT), its soluble lumenal homolog. Further deglucosylation of the oligosaccharides by GII liberates the glycoproteins from their CNX/CRT anchors. The glycans are then reglucosylated by the UDP-Glc:glycoprotein glucosyltransferase, and thus recognized again by the lectins, only when linked to misfolded protein moieties as this enzyme behaves as a sensor of glycoprotein conformations. The deglucosyl-ation-reglucosylation cycle continues until proper folding is achieved. The lectin-monoglucosylated oligosaccharide interaction is one of the alternative ways by which cells retain not properly folded glyco-proteins in the endoplasmic reticulum and although it decreases the folding rate, it increases folding efficiency, prevents premature glycoprotein oligomerization and degradation and suppresses formation of non-native disulfide bonds.

PL.3

Galectin-1 signaling in human leukocytes

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During inflammation leukocytes adhere to activated endothelium through interactions of selectins with specific glycoconjugates. Activated leukocytes then extravasate from the blood into tissue, where they turn over in part by macrophage-mediated phagocytosis. Our studies demonstrate that galectin-1 (dGal-1) may induce phagocytosis of activated leukocytes. dGal-1 potently induces changes in membrane and nuclear morphology in desialylated HL-60 and MOLT-4 cells, and in activated neutrophils, but much less so in resting neutrophils. dGal-1 treatment exposes annexin V binding sites, suggesting redistribution of phosphatidylserine to the outer leaflet of the plasma membrane. This induction by dGal-1 results in neutrophil recognition and phagocytosis by activated macrophages *in vitro*. Binding and induction by dGal-1 is completely inhibited by haptenic sugars. Induction also requires dimerization of dGal-1, since monomeric forms of Gal-1 bind to cells but fail to induce. Binding of dGal-1 to desialylated HL-60 cells induces a sustained Ca^{2+} influx that involves extracellular Ca^{2+}. Binding of dGal-1 to neutrophils induces a transient Ca^{2+} flux that requires both intracellular and extracellular Ca^{2+}. While these membrane changes induced by dGal-1 share some features with those seen in cells undergoing apoptosis, dGal-1-treated MOLT-4 and HL-60 cells do not exhibit changes in caspase activation or mitochondrial enzymes, fail to show DNA fragmentation, and the cells grow normally. In addition, the induction of annexin V-binding sites in dGal-1-treated cells is reversible after removal of dGal-1. We termed this dGal-1-induced process "adpararesis", to signify preparation of leukocytes for phagocytosis and turnover. Overall, these studies highlight how carbohydrate-binding proteins and specific glycoconjugates may regulate cell-cell interactions and cellular signaling.

PL.4

Congenital Disorders of Glycosylation: A road more traveled

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Glycoconjugate biosynthesis and recognition occupy some 2-3% of human genes. Human Congenital Disorders of Glycosylation (CDG) provide insight into diverse functions of N-linked glycans, and not surprisingly, patients with these disorders have multi-systemic pathologies. To study these disorders, we use well-defined yeast strains, mammalian cell lines, and mice with single defects in glycosylation in controlled environments. The conditions and genetic backgrounds are not so easily controlled in human populations.
About 30-40 genes are needed to synthesize and transfer the lipid-linked oligosaccharide (LLO) precursors to proteins on the ER assembly line. Excess LLO is degraded to avoid over-glycosylation, while on the other hand, deleterious mutations in rate-limiting glycosylation steps produce CDG. Some glycosylation-compromising alleles are relatively common in the population. Why? When combined with environmental stresses, e.g., infectious disease and poor nutrition, could such alleles have additive effects that compromise glycosylation? Based on recent data, we hypothesize and that CDG may be more widespread than we now appreciate. Viral pathogens and their human hosts both require protein N-glycosylation for survival, and frequent CDG mutations in the population may give heterozygotes a selective advantage, but insufficient glycosylation leads to CDG. (Supported by NIDDK 55615 and the CDG Family Network Foundation).

PL.5

Glycoconjugate ligands and regulation of selectin-mediated cell adhesion

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Selectin-mediated cell adhesion is involved in the routine homing of lymphocytes, inflammatory mobilization of leukocytes and tissue infiltration of malignant cells. Glycoconjugate ligands for selectins play the principal roles in the regulation of selectin-mediated cell adhesion. Two kinds of glycoconjugate ligands for selectin are noted in humans, one is conventional sialyl Le^x, and the other is sulfated sialyl Le^x as represented by sialyl 6-sulfo Le^x. Sialyl 6-sulfo Le^x primarily mediates routine homing of leukocytes, while conventional sialyl Le^x is preferentially involved in the recruitment of leukocytes to inflammatory lesions. Sialyl 6-sulfo Le^x is expressed on high endothelial venules of lymph nodes, Peyer's patches and appendices where it mediates homing of naïve T cells or gut-homing helper memory T cells. It is constitutively expressed also on a small subset of resting helper memory T cells, where it mediates homing of the cells to the skin. In contrast, conventional sialyl Le^x is expressed on granulocytes, monocytes and activated lymphocytes, extravasation of which is closely related to inflammation. Expression of conventional sialyl Le^x is regulated by transcription of the fucosyltransferase VII gene. Its 5'-regulatory region is equipped with CRE-consensus motif, GATA motif and MZF-1-binding site, which are involved in cell lineage-specific or stimulation-dependent expression of sialyl Le^x. Expression of the sulfated ligand is regulated by sulfation as well as fucosylation. Selectin-binding activity of sialyl 6-sulfo Le^x on human lymphocytes was found to be also regulated by a post-translational modification of its sialic acid moiety leading to the formation of cyclic sialyl 6-sulfo Le^x, and this would prevent excessive accumulation of leukocytes in the routine homing process.

PL.6

Investigations of the interactions between carbohydrates and proteins: A three-dimensional view by using NMR spectroscopy

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Carbohydrate-protein interactions are involved in a range of biological mechanisms. It is obvious that a detailed knowledge of the structure and dynamics of carbohydrates both free and bound to proteins is indeed relevant. In this context, different examples of the application of NMR as a tool to study protein-carbohydrate interactions in solution will be presented. In particular, cases of molecular recognition of galactose-containing saccharides by three different proteins (ricin-B, galectin-1, *E. coli* β-galactosidase) will be shown. It will be demonstrated that the same saccharide mimetic (C-lactose) is recognised by the proteins in three distinct conformations. Interestingly, the bound conformations of lactose to ricin-B, and to an inactivated *E. coli* β-galactosidase are different to those of C-lactose. On the other hand, the conformers of Galβ1,2Xyl, Galβ1,3Xyl, and Galβ1,4Xyl bound by galectin-1 and *Viscum album* agglutinin lectins are similar.
In a different context, the study by NMR of the specific interaction of natural and synthetically engineered hevein domains with GlcNAc oligomers will be described. The importance of mutations of key aromatic residues (Trp, Tyr, Phe, naphtalene) at the binding site will be presented from both the structural and energetic viewpoints. From the biological significant of hevein domains, it will be shown that recognition of chitin by these domains is a dynamic process, not restricted to the binding of the non-reducing end of the polymer, as previously thought. Thus, high affinity binding to a number of protein molecules is allowed, depending on polymer chain length, and therefore, the biological process is multivalent.

PL.7

News on polysialic acids: Biosynthesis and physiological importance

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α2,8-linked polysialic acid (PSA) is a unique posttranslational modification of the neural cell adhesion molecule (NCAM) and one of the best characterized factors correlated with neural plasticity. In addition, PSA has attracted considerable attention as a tumour marker. Understanding PSA-biosynthesis and expression would largely impact our basic knowledge of cellular and synaptic plasticity and could provide a new platform for the development of drugs, suited to influencing PSA-levels in pathophysiological situations. Two closely related polysialyltransferases, ST8SiaII and IV, are responsible for the polysialylation of NCAM. Our current work aims at understanding the physiological role and structure-function-relationships of these enzymes. Genetic models have been generated that allow (1) the dissociation of specific functions for ST8SiaII and IV *in vivo* and (2) the identification of amino acid residues that are crucial for the formation of active enzymes. Mice lacking ST8SiaIV, develop normal at macroscopic level, but are completely negative for PSA in adulthood. Loss of PSA is associated with an age dependent decrease in synaptic plasticity.
In order to find out how the activity of polysialyltransferases depends on a self-modification reaction called autopolysialylation, we generated glycosylation variant polysialyltransferases by either site directed mutagenesis or heterologeous expression of proteins in insect cells. Results obtained in both systems suggest that auto-polysialylation is prerequisite for NCAM-polysialylation.

PL.8

Function of chitin oligosaccharides: signal molecules involved in plant and animal organogenesis

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Lipo-chitin oligosaccharides (LCOs) are signal molecules, which were discovered as a result of the study of the root nodulation process in leguminous plants. Our recent results indicate that LCOs are representatives of a general class of chitin oligosaccharide (CO) signal molecules involved in plant and animal morphogenesis For example, we have demonstrated, using microinjection studies, that the COs are active in the development of organs in plant roots and zebrafish [1,2]. Modifications of the CO backbone modify the activity of these signal molecules. For instance, fucosylation is essential for activity on some plant species and abolishes activity in Zebrafish development. The proteins involved in the synthesis of the COs and fucosylated derivatives are studied in detail. They comprise the NodC and DG42 proteins, which are processive *N*-acetylglucosaminyltransferases [3], and NodZ protein which is a fucosyltransferase involved in the additon of fucose to C6 of Cos [4]. The latest data on the function of chitin oligosaccharides in Zebrafish embryogenesis will be presented.

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PL.9

Novel features of carbohydrate recognition by lectins

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Legume lectins ability to detect subtle variations in carbohydrate structures have made them paradigms for carbohydrate recognition. Their binding sites is made up of four highly conserved loops. However, the molecular basis of as to how a conserved set of residues allow for an overwhelming range of specificities in them was poorly understood. Our analyses showed that the remarkable repertoire of legume lectins emanates from their ability to use a conserved set of residues from loop A and B for hydrogen bonding with a distinct pair of sugar hydroxyls while utilizing variability in other regions (loop C and D) to achieve specificity. Based on these concepts peanut agglutinin was imparted exquisite specificity for tumor associated T-antigen. While the primary specificity of lectins for sugars is dictated by the orientation of the hydroxyl groups of the former around a pyranose ring, the stability of the complexes thus formed are modulated by water & water mediated hydrogen bonds, hydrophobic interactions, C-H...X type of hydrogen bond etc. Posttranslational modification(s) of lectins and their oligomerization, steric factors, valency, conformational changes etc. in the carbohydrate ligand influence strikingly their specificities.

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Invited lectures

KN.1

Glycosylation-dependent anti-inflammatory properties of α_1-acid glycoprotein

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α_1-Acid glycoprotein (AGP) is an acute-phase glycoprotein that has 5 N-linked glycans. Various distinct AGP-glycoforms occur in plasma that differ in degree of branching and α3-fucosylation of the glycans, and thus in the expression of sialyl Le^X groups. Inflammation induces changes in the relative occurrence of the AGP-glycoform, which are strictly dependent on the pathophysiological condition as determined by cytokines and hormones. To approach the functional consequences of these variations on anti-inflammatory properties of AGP, specific AGP-glycoforms were isolated from control and patient sera suffering from trauma, rheumatoid arthritis and other chronic diseases. In addition, polylactosamino-expressing and non-fucosylated glycoforms were obtained from neutrophilic granulocytes and seminal fluid, respectively. Increased α3-fucosylation and the presence of sialyl Le^X groups strongly increased the ability of AGP to inhibit the complement activation. These actions were also dependent on the degree of branching of the glycans and the presence of polylactosaminyl groups. The results suggest that the inflammation-induced changes in glycosylation of plasma AGP augment its anti-inflammatory potencies, but that this effect will differ under acute *versus* chronic inflammatory conditions.

KN.2

Carbohydrate recognition of cytokines modulates physiological activities

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On the basis of the mechanisms of carbohydrate recognition involved, cytokines have been grouped into three types to date. It has been determined that growth factors recognize glycosaminoglycans. Recently, we found that mannose-6-phosphodiester in GPI-anchored glycoproteins were recognized by IL-1β [2], TNF-α, and lymphotoxin and the addition of mannose-6-phosphate inhibited TNF-α induced apoptosis using U-937 cells or IL-1β induced cell proliferation using D10G4.1 cells. Furthermore, high-mannose type glycans with five or six mannose residues were recognized by IL-2, IL-5, and IL-6. We show you using IL-2 as an example how the carbohydrate recognition contributes to the physiological function. Addition of high-mannose type glycans inhibited not only IL-2-dependent CTLL-2 cell proliferation but also the phosphorylation of the related tyrosine kinases. Among a high affinity complex including IL-2-IL-2 receptor-α, -β, -γ subunits, only the IL-2Rα subunit was stained with high-mannose type glycan specific *Galanthus nivalis* agglutinin. These results suggest that dual binding of IL-2 to both a $Man_5GlcNAc_2$ moiety and specific peptide sequence in the IL-2 receptor α serves to trigger the formation of the high-affinity complex, leading to cellular signaling [2].

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KN.3

Heparan sulfate biosynthesis and the tumor suppressor EXT gene family

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Heparan sulfate (HS) plays critical roles in developmental and pathological processes. Recent studies have indicated that the human tumor suppressors EXT1 and EXT2 are the bifunctional glycosyltransferases essential for HS chain polymerization [1]. We characterized truncated soluble forms of the recombinant proteins, encoded by three homologous *EXT*-like genes [1,2]. Using a synthetic substrate, EXTL2 showed the enzyme activity of α1,4GlcNAc transferase I, the key enzyme that initiates the HS synthesis. EXTL1 transferred α1,4GlcNAc to *N*-acetylheparosan oligosaccharides that represent growing HS chains. EXTL3 transferred α1,4GlcNAc to both acceptors. Hence, EXTL3 is most likely involved in both chain initiation and elongation, whereas EXTL1 is likely involved only in the chain elongation of HS. Thus, the acceptor specificities of the five family members are overlapping, but distinct from each other except for EXT1 and EXT2 with the same specificity. It has now been clarified that all the five cloned human *EXT* gene family proteins harbor glycosyltransferase activities, which probably contribute to the synthesis of HS and probably its analog heparin.

References

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KN.4

Mammalian chitin hydrolysing and binding proteins

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Chitin is an abundant glycopolymer, being present in coatings of many species ranging from fungi to arthropods. Until quite recently it was generally thought that vertebrates lack chitinases. This view has been completely changed by the discovery in man of the enzyme chitotriosidase, a functional chitinase. Chitotriosidase is selectively expressed by activated phagocytes and can serve as diagnostic marker for some pathologies. It has in recent years become apparent that in man an extensive family of chitinase(like) proteins is expressed. The genetic aspects, molecular properties, and antifungal action of chitotriosidase, as well as its diagnostic and potential therapeutic applications will be described. The properties of a very recently identified, distinct mammalian chitinase (amcase) are discussed. Finally, the features of other homologous proteins, that are catalytically inactive and have evolved into chitin binding lectins, will be addressed.

KN.5

Interactions of the myelin-associated glycoprotein with glycoconjugates of the nervous system

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Myelin-associated glycoprotein (MAG) is expressed by myelinating glial cells in the central and peripheral nervous system of vertebrates. It is involved in the formation and maintenance of myelin and inhibits or promotes neurite outgrowth depending on the differentiation stage and type of neurons. MAG is a member of the siglec family and binds to sialic acid residues of cell surface glycoconjugates on cells from the central nervous system including neurons, astrocytes and microglia. Several glycoproteins could be isolated from different cell types by affinity precipitation with MAG-Fc followed by specific elution with a synthetic sialic acid analogue. Whereas from neuroblastoma and primary cultured neurons similar glycoprotein patterns were obtained, different glycoproteins were isolated from glial cells. The glycoproteins isolated from neurons, astrocytes and microglia were further analysed by peptide microsequencing and immunoblotting indicating interactions of MAG with extracellular matrix proteins.

Reference

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KN.6

Structure and function of N-glycan processing α1,2-mannosidases

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Class I α1,2-mannosidases (MANases) consist of two groups with distinct enzymatic properties. The first includes *S. cerevisiae* and human ER MANases that form $Man_8GlcNAc_2$ (Man8) isomer B from $Man_9GlcNAc_2$. The second includes mammalian Golgi MANases, that trim $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$ *via* Man8 isomers A/C. The crystal structures of yeast (group I) and *Penicillium citrinum* (group 2) MANase were compared. Each has an $(\alpha\alpha)_7$ barrel structure with one side of the barrel plugged by a β-hairpin. The active site is located in the centre of the barrel at the bottom of a ~15Å cavity. In yeast, an N-glycan from one molecule extends into the barrel of an adjacent molecule as an enzyme-product complex. Mutation of R273, which stabilizes the oligosaccharide, to L present in mammalian Golgi MANases, changes the specificity of the yeast MANase. Differences occur between the yeast and fungal enzymes near the oligosaccharide binding site where replacement of R273 by G, enlarges the cavity of the barrel. Models of Man8 isomers indicate that the active site of the fungal enzyme can accommodate various conformations of Man8 isomers A, B or C whereas the ER MANase accommodates only one Man8 isomer B conformer. (Support: NIH Grant GM31265 and CIHR)

KN.7

Deciphering regulation of mucin-type O-glycosylation

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Mucin-type O-glycosylation is initiated by a large homologous family of polypeptide GalNAc-transferases. Nine members of this GalNAc-transferase family have been characterized so far, and data clearly indicate that they have different functions. *In vitro* analysis of the kinetic properties of recombinant GalNAc-transferase isoforms show that they have distinct acceptor substrate specificities and several isoforms show selective specificity for partial GalNAc-glycosylated substrates. The glycosylation dependent activity found for some GalNAc-transferase isoforms appears to be directed by a lectin domain found in the C-terminae of most GalNAc-transferases. This effect appears to provide a follow-up function which is suggested to play a role in directing and controlling pattern and density of O-glycan attachments. An update of studies aimed at defining "rules" for the initial O-glycan attachment process and the following O-glycan processing step will be given.

KN.8

Prion glycoprotein: structure, dynamics and roles for the sugars

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Prion protein contains two N-linked glycosylation sites and a glycosylphosphatidylinositol (GPI) anchor. The large size of the N-linked sugars, together with their dynamic properties, enable them to shield two orthogonal faces of the protein almost completely. Thus the sugars can protect large regions of the protein surface from proteases and from non-specific protein-protein interactions. Major questions relate to the relevance of the glycoform distribution (defined by glycan site occupancy as well as site heterogeneity) to location, strain type and disease transmission. Glycan analysis has shown that prion protein contains at least 52 different sugars and that these consist of a sub-set of brain sugars. PrP^{Sc} from scrapie infected Syrian hamster brain contains the same set of glycans as the cellular form, PrP^{C}, but contains a higher proportion of tri- and tetra-antennary sugars. This may be attributed to a decrease in the activity of GnTIII.

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KN.9

Tissue-specific regulation of core-2 β6GlcNAc transferase and its functional aspects

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We reported that an autosomal mouse gene, *Gsl5*, controls the expression of core-2 β6GlcNAc transferase in a kidney proximal tubule cell-specific manner. The β6GlcNAc transferase is responsible for the production of a branch structure of GlcNAcβ1-6(Galβ1-3)GalNAcβ1- and α1- in glycosphingolipids and glycoproteins, respectively. Histological experiments and Western blotting with a monoclonal antibody recognizing the structure of Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-3)GalNAc-α1- demonstrated the presence of a glycoprotein which localizes to membrane vesicles just below the apical microvillous membrane of proximal tubule epithelial cells, is recovered in a microsomal fraction, and behaves like a molecule with 400 kDa molecular mass. The glycoprotein appears to be a protein known as megalin. A polyclonal antibody raised against a KLH-coupled 17-mer peptide, the sequence of which is identical to a part of mouse megalin, was able to stain the 400 kDa glycoprotein by Western blotting, indicating that the glycoprotein is in fact megalin. We are in the process of functional assay of megalin and analysis of its functional modulation. A transgenic mouse experiment demonstrated that a 150-kb long BAC clone in-cluding the β6GlcNAc transferase gene can rescue a *Gsl5*-defective phenotype.

Reference

Sekine M *et al* (2001) *Eur J Biochem* **268**:1129-1135

KN.10

Mammalian heparanase: Molecular properties and involvement in tumor metastasis and angiogenesis

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Heparan sulfate proteoglycans (HSPGs) play a key role in the self-assembly, insolubility and barrier properties of basement membranes and extracellular matrices. Mammalian heparanase, endo-β-D-glucuronidase, is synthesized as a latent ~65 kDa protein that is processed at the N-terminus into a highly active ~50 kDa form. The heparanase mRNA and protein are preferentially expressed in metastatic cell lines and human tumors. Overexpression of the heparanase cDNA in low-metastatic tumor cells conferred a high metastatic potential in experimental animals. The heparanase enzyme also releases ECM-resident angiogenic factors and its overexpression induces an angiogenic response *in vivo.* Heparanase may thus facilitate both tumor cell invasion and neovascularization, both critical steps in cancer progression. The enzyme is also involved in inflammation and autoimmunity. Heparanase inhibitors markedly inhibit tumor growth, metastasis and autoimmune disorders. Studies are underway to elucidate the involvement of heparanase in normal processes such as implantation, embryogenesis, tissue repair, immune surveillance and HSPG turnover. Heparanase is the first functional mammalian HS-degrading enzyme that has been cloned, expressed and characterized. This may lead to identification and cloning of other GAG degrading enzymes, toward a better understanding of their involvement in normal and pathological processes.

KN.11

New routes in modeling complex glycans and their interaction with protein

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Because of the flexibility of the glycosidic linkages and the large number of conformations that can be adopted by some oligosaccharides such as N-glycans, special strategies have to be used for defining their conformational behavior. Energy force fields developed for carbohydrates, together with methods for exploring the conformational space have to be used for modeling oligosaccharides. Examples will illustrate the approaches that we developed for modeling flexible oligosaccharides in the binding site of protein receptors such as lectins or glycosylhydrolases. Plant lectins recognize specifically complex oligosaccharides. In addition of the interactions occurring in the primary binding site, the oligosaccharides can establish additional contacts in secondary binding site at the protein surface. *Maackia amurensis* hemagglutinin recognizes specifically a disialylated tetrasaccharide and we could predict that both NeuAc residues play a direct role in the interaction. Class I α1,2-mannosidases from various origins display different hydrolysis patterns towards N-glycans. Conformational analysis of the branched oligosaccharide in the binding site of the enzyme help rationalizing the observed specificity.

Reference

Imberty A and Pérez S (2000) *Chem Rev* **100:**4567-4588

KN.12

Structural basis for neutralization of Shiga-like toxins by synthetic multivalent carbohydrate ligands

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Crystal structure data for the Type 1 Shiga like toxin (SLT-1) from *E. coli* O157 have been used to design STARFISH, a decavalent, water soluble and inhibitor of toxin binding to the trisaccharide of Gb_3. The mode of action of the inhibitor was determined from a crystal structure of the STARFISH-toxin complex and by FTICR mass spectrometry studies of non-covalent STARFISH-SLT-1 complexes.
The therapeutic efficacy of Starfish, has been evaluated in a murine model. One hundred percent of mice (10/10) injected with SLT-1, became lethargic, started to loose motor coordination and required euthanasia after 3 to 4 days. However, 100% of mice injected with an emulsion of SLT-1 and Starfish at a molar ratio of 10,000:1 (Starfish:Stx1), 25 μg/g body weight displayed no evidence of SLT-1-mediated toxicity up to 2 weeks from the time of challenge. These data demonstrate the potential *in vivo* efficacy of Starfish in preventing HUS in patients infected with enterohemorrhagic *E. coli*.

Reference

Kitov PI *et al* (2000) *Nature* **403**:669-672

KN.13

Synthesis of glycosylphosphatidylinositols in yeast and identification of potential targets for antifungal agents

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The assembly pathway for the Manα1,2Manα1,6Manα1,4-GlcN-α1,6Ins-phospholipid core of the GPI precursor that is transferred to protein in all eukaryotes is highly conserved. However, side-branches can be added to this core, and their relative importance for completion of the pathway and for cell viability varies between species. Our studies of GPI assembly in yeast have revealed that late steps in the pathway differ between yeast and mammals. In yeast, addition of a fourth, α1,2-linked Man to the third Man of the GPI precursor is carried out by the essential Smp3 protein, and precedes addition of phosphoethanolamine (EthN-P) to Man-3 by the Gpi13p EthN-P transferase. In contrast, addition of a fourth Man to GPI precursors is at best rare in mammalian cells and EthN-P can be added to Man_3-GPIs. Because addition of a fourth Man is essential in yeast, but apparently not in mammals, Smp3p is a potential antifungal target. The yeast Gpi13 protein could also be inhibited selectively, because, in contrast to its mammalian counterpart, it requires a Man_4-GPI as acceptor. In baker's yeast, a block in GPI assembly leads to defects in cell wall assembly and morphogenesis and is lethal. This is also the case for the human pathogen *Candida albicans*: deletion of both copies of *CaGPI3* (which encodes the catalytic subunit of GlcNAc-PI synthase) is lethal, and heterozygous *CaGPI3* disruptants have cell wall defects. *C. albicans* has an *SMP3* gene, disruption of which is in progress. (Supported by NIH GM46220 and the Burroughs Wellcome Fund).

KN.14

X-ray crystal structure of rabbit *N*-acetylglucosaminyltransferase I: Enzyme mechanism and a new protein superfamily

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The medial-golgi enzyme UDP-*N*-acetylglucosamine:α-3-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase I (GnT I, EC 2.4.1.101) is a key enzyme in the asparagine-linked glycosylation pathway; its action serves as the gateway from oligomannose to complex and hybrid glycans. We have solved the x-ray crystal structure of a soluble fragment containing the catalytic domain of rabbit (*Oryctolagus cuniculus*) GnT I in both the presence and absence of UDP-GlcNAc. The structure is formed by two mixed β-sheets: (i) an N-terminal 8-stranded β-sheet containing the acceptor and Mn^{2+}/UDP-GlcNAc binding sites, and (ii) a C-terminal 4-stranded β-sheet with no obvious function. Both β-sheets are flanked by α-helices. The structure provides insight into the inverting catalytic mechanism, as well as an explanation for the observation that the reaction proceeds in an ordered sequential fashion. The structure also provides support for the suggestion that the glycosyltransferase reaction shares mechanistic similarity with that shown by glycosidases. Comparison with other glycosyltransferase structures, in conjunction with sequence analysis, suggests a common structural core likely to be found among members of a number of glycosyltransferase families.

KN.15

Specific protein assembly on the engineered glycolipid membranes

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Some photopolymerizable sphingoglycolipids and their mimetics were synthesized by means of chemical and enzymatic synthetic strategy. These materials showed excellent self-assemblying capacity to form stable monolayers and they can be converted into the glycopolydiacetylene films (LB membranes) by UV irradiation. The image of the adsorbed lectins on the glycolipid LB membranes were observed by using atomic force microscopy and exhibited the formation of the specific protein arrays such as networks, dendrites and microdomains (patches). Interestingly, the morphology of the protein arrays greatly depends on the structure of the aglycons (linkers) in addition to the structure or the density of sugar moiety. The present results demonstrate that molecular imprinting technique on the basis of engineered glycolipid thin films can be applied for the controlled assembly of protein arrays with specific morphology.

References

Nishimura S-I and Yamada K (1997) *J Am Chem Soc* **119**:10555-10556
Nagahori N and Nishimura S-I (2001) *Biomacromolecules* **2**:22-24

KN.16

Biosynthesis of the GPI-anchored lipophosphoglycan of *Leishmania*

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The glycocalyx of *Leishmania* parasites is composed primarily of a GPI-anchored polysaccharide called lipophosphoglycan (LPG), a critical virulence factor. The *L. donovani* LPG consists of a polymer of repeating Gal(β1,4)Man(α1-PO_4) units (avg. n=15-30) linked to the GPI anchor. In collaboration with Dr. Stephen Beverley (Washington Univ.), we have shown that LPG biosynthesis is an attractive parasite system for biochemical genetics as *lpg*$^-$ mutants can be rescued by genetic complementation, thereby permitting identification of genes involved in its assembly. One gene was isolated by complementation of the *L. donovani* mutant OB1, which is unable to galactosylated the first repeat unit. Functional rescue of OB1 resulted in cloning the gene *LPG3*, a *Leishmania* homologue of the mammalian chaperone GRP94. Characterization of OB1 cells revealed that in contrast to GRP94 in mammals, the role of LPG3 is restricted to LPG repeat unit assembly since functions such as N-glycosylation and protein secretion are normal in mutant cells. Another gene (*LPG4A*) was isolated by complementation of the JEDI mutant that is unable to elongate the repeat units due to a defect in the elongating mannosylphosphoryltransferase step. Thus, isolation and characterization of genes, such as these two, may help to provide a comprehensive understanding of the biochemical and genetic aspects of LPG biosynthesis. Conceivably, this detailed information may lead to establishment of a new, effective parasite-specific therapeutic regimen for the treatment of leishmaniasis.

KN.17

Expression and prognostic value of sialyltransferases in breast cancer

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The human genome encodes probably more than 20 different sialyltransferases (ST) and to date, 15 different human ST cDNAs have been cloned and characterised. A Multiplex RT-PCR method has been used to monitor the expression of the six Gal-specific ST in human breast tumours. We have shown that ST3Gal III, the enzyme involved in sialyl Lewis^a synthesis, was the most expressed ST and that a high ST3Gal III / ST6Gal I ratio was associated with a poor prognosis and a shorter overall survival of patients with node-negative breast cancer indicating that assessment of tumoral ST expression could be a useful prognostic marker for these patients.
Screening EST and genomic databases have revealed at least six different human ST6GalNAc. Three of them, involved in the biosynthesis of O-glycans, namely hST6GalNAc I, II and IV, were studied in detail. Their gene expression has been studied in various human tissues and in various human cancer cells. These enzymes slightly differ in their substrate specificity and based on *in vitro* assays, ST6GalNAc I and II are considered as the main enzymes responsible for the synthesis of sialyl-Tn.

KN.18

Ganglioside-enriched membrane domains: composition, properties, organization and functional role

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The recent years have been characterized by a huge interest in the structure and function of mammalian cell membrane lipid domains. The interest in this subject grew further, when their participation in important membrane-associated events such as signal transmission, cell adhesion and lipid/protein sorting was postulated. A common feature of cell membrane domains is their peculiar lipid composition, being they enriched in glycosphingolipids, sphingomyelin and cholesterol. A series of theoretical considerations and several experimental data suggest that glycosphingolipids play an important role in the formation and function of membrane domains. Within this lecture, the involvement of gangliosides into biogenesis, structure and function of domains is discussed at the light of their strong amphiphilic nature and of their peculiar chemical features. These features differentiate gangliosides from other lipids in the membrane, allowing either self-interaction or interaction with other membrane components and external ligands. Due to these interactions, gangliosides undergo lateral phase separation, segregation, and therefore form *core* domains within the membrane; ganglioside domains constitute the nucleation point that allows co-segregation of other lipids and proteins in a complex domain; finally, gangliosides confer dynamic properties on domains, that are essential to the modulation of cell functions.

Ground-breaking lectures

GB.1

Identification of an O-GlcNAc binding motif in nucleo/cytoplasmic proteins

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Since the early 80's it has been clear that many intracellular proteins are modified by O-linked β-*N*-acetylglucosamine (O-GlcNAc). Site mapping studies have indicated that this modification often occurs at or near sites of phosphorylation, leading to the attractive hypothesis that there is a reciprocal relationship between phosphorylation and glycosylation. We have recently shown that the essential splicing factor ASF/SF2 is modified by O-GlcNAc and that the glycosylated species is predominantly cytoplasmic. Analyses of proteins that bind ASF/SF2 have indicated that MA32 (p32/gC1q-R/TAP) binds Ser-β-O-GlcNAc preferentially to Thr-β-O-GlcNAc immobilized on agarose, but NOT β-O-GlcNAc alone. MA32 is an attractive target as an O-GlcNAc binding protein as it binds a multitude of other O-GlcNAc modified proteins, including the Lamin receptor and TF IIb. More importantly, MA32 joins a growing family of intracellular proteins with a known amino acid motif for binding hyaluronan (HA), repeating units of [-4)-β-D-GlcA-(1-3)-β-D-GlcNAc-(1-]. We report here our recent studies, indicating which of these HA domain proteins are able to bind O-GlcNAc and the functional implications of these interactions. Isolation of an O-GlcNAc binding motif, analogous to phospho-amino acid binding domains such as SH2 domains, will greatly enhance our understanding of the role of O-GlcNAc in cellular processes. (Supported by NIH CA42486 and HD13563 to GWH).

GB.2

New insights into the structure and function of eukaryotic peptide:*N*-glycanase

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Peptide:*N*-glycanase (PNGase) is an ubiquitous deglycosylating enzyme widely distributed in eukaryotes. It has been suggested that PNGase activity is involved in a ubiquitin-proteasome pathway to degrade glycoproteins. We identified the gene encoding a cytoplasmic PNGase (Png1p) in *S. cerevisiae* [1]. Further, it was found that PNGase interacts with a DNA repair protein, Rad23p in yeast and mammalian cells. Strikingly, Rad23p was shown to mediate interaction between Png1p and the 26S proteasome, providing evidence of physical interaction between the deglycosylating enzyme and the proteasome [2]. Database analysis revealed that Png1p and its homologues can be classified as members of a "transglutaminase-like superfamily" which share a conserved amino acid sequence surrounding a catalytic amino acid triad (Cys, His, Asp) [3]. These residues were conserved in all Png1p homologues and found by mutagenesis to be essential for catalytic activity. Since thus far proteins in this superfamily are responsible for either formation or hydrolysis of amide bonds, cytoplasmic PNGase, an amidase, can be regarded as a member of the "transglutaminase-like" superfamily.

References

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[2] Suzuki T *et al* (2000) *J Biol Chem* **276**: in press
[3] Makarova *et al* (1999) *Prot Sci* **8**:1714-1719

Symposium Topics

C1. Analytical methods in glycoscience

C1.1

Analyses of human milk glycoproteins by hemagglutination inhibition and Western blotting using diverse lectins

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Human milk contains various glycoproteins which differ in molecular weight as well as in amino acid and carbohydrate composition. Their terminal saccharides include mannose, galactose and its derivatives, fucose, *N*-acetylglucosamine, etc. Such compounds may contribute to protection of the newborn from infections. They may also be valuable for blocking microbial and cancer cell adhesins – for preventing adult infections and metastatic invasion. Therefore, their isolation is desired. Usage of lectin affinity chromatography is of utmost advantage for this purpose due to their selective sugar specificity. Preparations of human milks, at various breast feeding stages, were examined with a series of lectins in hemagglutination inhibition test and Western blot analyses (peroxidase-labeled lectins). The lectins used were WGA (chitotriose/ sialic acid), Con A (Man/Glc), *Maclura pomifera* lectin (MPL, GalNAc/Gal), *Pseudomonas aeruginosa* lectins PA-IL and PA-IIL (interacting with Gal and Fuc/Man, respectively), and *Aplysia* gonad lectin (AGL, GalA/Gal). Both the hemagglutination inhibition and the Western blots nicely showed the differential selective interactions of the examined lectins with the different milk glycoproteins. The lectins sharply differed in their preferential inhibition by the whole milk samples as well as in the tagging of the individual milk components. The results obtained enable programming of a sequential differential high quality separation of the desired glycoproteins.

C1.2

Differential analyses of avian egg white glycoproteins using a series of diverse lectins

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Avian egg whites contain a battery of glycoproteins differing in structure and carbohydrate composition, associated with diverse interactions with different lectins. Generally, wheat germ agglutinin (WGA) and Con A are used for industrial preparations of hen egg white glycoproteins. The present communication describes a comprehensive, comparative study of chicken, quail and pigeon egg white glycoproteins with various lectins, including the latter two lectins, galactophilic lectins [from *Aplysia* gonad (AGL), *Erythrina corallodendron* (ECorL), peanuts (PNA) and *Pseudomonas aeruginosa* (PA-IL)], and fucose-binding lectins [from *Ulex europaeus* (UEA-I), *Ulva lactuca* (ULL) and *P. aeruginosa* (PA-IIL), which also binds mannose]. Both hemagglutination inhibition and Western blot analyses indicated differential selectivity of these lectins. The chicken egg white glycoproteins interacted most strongly with WGA, followed by Con A >> AGL = PA-IIL >> ECorL. Their interactions with the other lectins were very weak. The quail glycoprotein order of affinities was: Con A > WGA = AGL = PA-IIL, while those of the pigeon interacted most strongly with AGL followed by PA-IL > WGA > Con A = PA-IIL. The results strongly imply the potential efficiency of the examined lectins for study of avian egg glycoproteins and their profound heterogeneity, for improved sequential high quality purification of the desired glycoproteins, and for quality control of the purified preparations.

C1.3

Determination of chondroitin/dermatan sulfates in brain of rats

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The role of glycosaminoglycans (GAG)/proteoglycans (PGs) in brain has been widely noticed and studied by many workers. In this study, we focused on chondroitin/dermatan sulfates (C/DSs) in the matrix of rat brain and established a method for the determination of C/DSs in the histological tissue sections on a glass slide: The serial polyester wax sections of brain were processed into cerebral-neocortex, hippocampus and cerebellum by a small surgical knife. The C/DSs in the tissue sections on a glass slide were digested by chondroitinase ABC and ACII. The resulting unsaturated disaccharides were determined by the reversed-phase ion-pair HPLC with fluorometric postcolumn derivatization using 2-cyanoacetamide as a reagent. This method was applied to the determination of C/DSs during the development of rat brain, and the structural characteristics of oversulfated C/DSs in the brain were examined. We also measured C/DSs in the brain of rats subjected to convulsant action of pilocarpine. Based on this section analysis, it was possible to determine C/DSs in a small local part in the tissue sections of hippocampus.

C1.4

Method of visualization of the surface carbohydrates of Mollicutes using plant lectins labelled by colloid gold

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The invasion capacity is one of the determining factors of pathogenicity of mollicutes. It consists in their ability to adhesion on the surface of macroorganism's cells and to grow and propagate there. The adhesion properties of pathogenic Mollicutes are determined by the presence in their glycocalix structure as well lectins as carbohydrate residues of surface glycopolymers. It is interesting to investigate the definite carbohydrate determinants on the surface of Mollicute cells. The method of visualization of the surface carbohydrates by electron microscopy was elaborated on the basis of the Horisberger and Rosset method. The plant lectins labeled by colloid gold are incubated with Mollicute cells for the complex forming and after washing are placing to the grids with formvar film with future microscopy. The units of colloid gold is a convenient electron-dense contraster of the surface carbohydrates since allows distinct determine the definite monosaccharide groups by the connections of specific lectins. This method permits also to investigate the localization of carbohydrate determinants and specific links between lectins and glycocalix of cells as well Mollicutes as other procariots.

C1.5

Analyses of N-linked glycans of recombinant DNA-derived human erythropoietins produced under different culture conditions: relationships between N-glycan structures and biological activities

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Eight preparations of recombinant DNA-derived human erythropoietins (rhEPO), which had been produced under various culture conditions, were analysed for their N-glycan structures and bioactivities. An HPLC strategy was used to map the N-glycans, consisting of the release of the N-glycans by PNGase F digestion, their fluorescent labelling with 4-aminobenzoic acid and their mapping by anion exchange and normal-phase HPLC. The sensitivity of the method permits its use for the analysis of small amounts (2-5 μg) of glycoproteins, as in the case of batches of low yield recombinant glycoproteins or of the dosage forms of therapeutic materials. The contents of biantennary and partially sialylated multiantennary glycans in the rhEPOs studied were found to be related to their in-vitro bioactivities, and, in preliminary studies probably also, inversely related to their in-vivo bioactivities. MALDI mass spectro-metry of N-glycan fractions also suggested the presence of acidic (phosphorylated/sulphated) oligomannose structures. However, this finding requires confirmation. The N-glycan mapping procedure described here is easy to perform and can be adapted to the equipment available in most laboratories.

C1.6

Ion-pair solid-phase extraction and reversed-phase HPLC analysis of nucleotide sugars from cell lysates

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A method for the extraction and separation of nucleotide sugars from cell lysates is described. We show that solid-phase extraction cartridges containing graphitized carbon can be used for the purification of nucleotide sugars, after which they are separated by high-performance liquid chromatography using a C18 reversed-phase column. Triethylammonium acetate buffer was used as a ion-pairing reagent for decreasing retention in colums containing graphitized carbon and for increasing retention in reversed-phase HPLC. Advantages of these techniques over more conventional sample preparation and analysis methods include selectivity, good separation of structurally similar sugar nucleotides, compatibility with rapid evaporative concentration, and possibility to automation. The new methods allowed monitoring the production of GDP-L-Fuc and GDP-D-Rha in yeast and their preparative purification. Nucleotide sugar profiles of bacterial cells were also analysed.

C1.7

N-Glycan structures from the major glycoproteins of pigeon egg white: Predominance of terminal Galα(1-4)Gal

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The presence of the galabiose (Galα(1-4)Gal) sequence in N-glycans in any animal is only rarely noted. We have demonstrated that the galabiose sequence is abundantly present in pigeon egg white glycoproteins. N-Glycans from major glycoproteins of pigeon egg white (ovotransferrin, ovomucoid, and ovalbumins) were enzymatically released and reductively aminated with 2-aminopyridine, separated, and structurally characterized by mass spectrometry and a three-dimensional mapping technique using three columns (DEAE, ODS and amide adsorption) of HPLC. Twenty-five major N-glycan structures, all of them hitherho unknown, were identified as pyridylamino derivatives. Of these, 13 were neutral, 10 were monosialyl and 2 were disialyl glycans. All N-glycans contain from one to four Galα(1-4)Galβ(1-4) sequences at the nonreducing terminal positions and are devoid of fucose residues. *N*-Acetylneuraminic acids were α(2-6)-linked only to β-galactose. The HPLC profiles of the N-glycans from 4 different glycoproteins were qualitatively very similar to each other, but not identical in the peak distributions.

C1.8

Carbohydrate electrophoresis on the DNA-sequencer: technology development and first applications

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We have adapted a standard ABI 377 DNA sequencer for the analysis of N-linked oligosaccharides. The detection limit is 10 fmol of APTS-labelled carbohydrate and the resolution is comparable to capillary electrophoresis. The sample preparation process has been optimised to make full use of the sensitivity offered by laser-induced fluorescence detection. All steps can now be performed in 96-well plates, allowing for medium-throughput analysis and anticipating the use of capillary-array based sequencers. A compatible procedure has been developed for the glycan analysis of microgram-quantities of SDS-PAGE separated proteins using a meltable polyacrylamide formulation.
This technology has been applied for the analysis of N-linked oligosaccharides present on serum glycoproteins of patients with different types of Congenital Disorders of Glycosylation (CDG). All types of CDG I investigated thus far result in an increased core-and branch fucosylation and a reduced amount of tri-and tetra-antennary N-glycans. Analysis of a patient with CDG IIa revealed the accumulation of monoantennary glycans, as expected. We anticipate that the method will be valuable for the analysis and elucidation of unknown types of CDG II. Further experience with the technology has been gained in the N-glycan analysis of submicrogram quantities of plant-produced antibodies, new recombinant variants of erythropoetin and in the evaluation of N-glycan bio-engineering in yeasts and fungi.

Reference

Callewaert N *et al* (2001) *Glycobiology* (in press)

C1.9

Modeling of the structure in aquous solution of the exopolysaccharide produced by *Lactobacillus helveticus* 766

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A method is described for constructing a conformational model in water of a heteropolysaccharide built up from repeating units, and is applied to the exopolysaccharide (EPS) produced by *Lactobacillus helveticus* 766. The molecular modeling method is based on energy minima, obtained from molecular mechanics calculations of each of the constituting disaccharide fragments of the repeating unit in vacuo, as starting points. Subsequently, adaptive umbrella sampling of the potential-of-mean-force is applied to extract rotamer populations of glycosidic dihedral angles of oligosaccharide fragments in solution. From these analyses, the most probable conformations are constructed for the hexasaccharide repeating unit of the EPS. After exploring the conformational space of each of the individual structures by molecular dynamics simulations, the different repeating unit conformations are used as building blocks for the generation of oligo- and polysaccharide models, by using a polysaccharide building program. The created models of the EPS produced by *L. helveticus* 766 exhibit a flexible twisted secondary structure and tend to adopt a random coil conformation as tertiary structure.

β-D-Gal*f*-(1→3)
\
[→3)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1→]$_n$

C1.10

Determination of lipid-bound sulfate by ion chromatography and its application to quantification of sulfolipids from kidneys of various mammalian species

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A variety of procedures have been developed for determining the sulfate ester content of various biomolecules. Recently, ion chromatography (IC), i.e. quantitation of ionic substances by ion conductimetry, is frequently applied for the determination of inorganic sulfate. We adopted non-suppressed-mode IC to the determination of the lipid- or glycolipid-bound sulfate released by acid hydrolysis after separation by anion-exchange chromatography, and found that the present method has the advantage of increased precision without interferences from other lipids. To minimize deterioration of the separation column, the lipophilic constituents in the acid hydrolysate were removed by a two-phase partition system of chloroform/ methanol/water containing an alkaline buffer. By the present method, the concentration of sulfolipids was determined in the kidney of mammals with various body mass. Sulfolipids were more concentrated in the kidney of smaller animals, which have higher urine concentrating activity per g body mass, supporting the hypothesis of the function of sulfolipids as an ion-barrier on the luminal surface of renal tubules.

Reference

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C1.11

Glycoform analysis of α_1-acid glycoprotein by capillary electrophoresis - Effect of heterogeneity of carbohydrate chains on glycoform separation

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α_1-Acid glycoprotein (AGP) is a plasma glycoprotein of 41 – 43 kD in molecular mass. Carbohydrates occupy approximately 45% of the whole protein, and attach to the protein core as the form of five N-linked glycans.
We could separate AGP into eight peaks by capillary electrophoresis using a chemically modified capillary in a buffer around their isoelectric point. We collected each glycoform peak and examined by MALDI-TOF MS. The peak showed characteristic molecular ions. Furthermore, we analyzed the carbohydrate chains released from AGP molecular species separated by anion-exchange chromatography, Con A and Cu(II)-chelating affinity chromatography after derivatization with 3-aminobenzoic acid.
The results indicate that capillary electrophoresis is a powerful technique for the glycoform analysis of sialic acid-containing glycoprotein. However, it is difficult to predict the changes of the ratios of respective carbohydrate chains during biological and clinical events. Thus, we have to examine both glycoforms of proteins and carbohydrate chains released from protein core for the accurate evaluation.

C1.12

Suppression of hyaluronic acid using 4-methylumbelliferone induces changes of glycosaminoglycans in mice

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Hyaluronic acid (HA) plays many roles in physiological events such as embryonic and fetal development, and also in pathophysiological status such as tumor growth. We have reported the induction of HA deficient extracelluar matrix by addition of 4-methylumbelliferone (4MU) to the medium of cultured human skin fibroblasts [1]. In this study, we injected 4MU sodium salt (4MUNa) in mice from the tail vein and quantified the concentration of 4MUNa in the whole blood by high-performance liquid chromatography (HPLC).
Glycosaminoglycans (GAG) were extracted from blood and organs, and then analyzed by HPLC [2]. Moreover, HA levels were exactly quantified by ELISA assay. As a result, serum HA decreased and tissue HA showed a slight decrease. Furthermore, other GAGs also showed small changes. This suggests that suppression of HA induces changes in other GAGs in mice.

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C1.13

Development and qualification of an assay measuring capping of β-linked galactose residues in glycoproteins

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Recombinant glycoproteins contain N- and/or O-linked oligosaccharide structures, which are either partially or fully capped with sialic acids. The clearance of glycoproteins through the hepatic asialoglycoprotein receptor depends on the level of exposed galactose on the oligosaccharides. This is a critical issue that affects the pharmacokinetics and bioavailability of many recombinant therapeutic glycoproteins. This report describes a convenient assay method for accurate measurement of the percent galactose residues capped with terminal sialic acid. The assay provides a direct assessment of the ratio of exposed versus total galactose residues in a therapeutic glycoprotein. The assay involves the release of the native "terminal" and "total" galactose residues from both N-and O-linked glycans of denatured glycoprotein using bovine testicular β-galactosidase (cleaves both terminal β-1,3 and β-1,4 galactose residues) and a mixture of β-galactosidase and sialidase, respectively. Digestion is performed at 37 °C for 3 hr in two tubes containing the same amount of protein. The released galactose residues from the two digests are analyzed by HPAEC-PED. The result is expressed as the peak area ratio of exposed galactose over total galactose and reported as %-uncapped galactose (exposed galactose area/ total galactose area X 100). The assay conditions, including the enzyme:protein ratio, pH, incubation time and enzyme source for complete release of galactose, were optimized using a complex glycoprotein (BHK-derived recombinant human FVIII). The assay was validated for accuracy, precision, repeatability, linearity, specificity and robustness. The assay, which is precise and accurate, can, in principle, be used with any therapeutic glycoprotein.

C1.14

Glycosylation analysis of α_1-acid glycoprotein using a lectin immunosensor technique

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Glycosylation of α_1-acid glycoprotein (AGP) is known to change in association with inflammatory diseases. Thus, glycosylation of AGP could be a potential diagnostic or prognostic marker. This study describes a surface plasmon resonance based affinity biosensor assay for quantification of concentration and glycosylation of AGP. AGP in cell culture supernatant, diluted EDTA plasma, or serum was analyzed directly in a BIACORE® 2000. AGP was captured on a sensor surface using immobilized antibodies. The response for captured AGP could be used to determine AGP concentration. Lectins were then injected and glycosylation of captured AGP was determined by measuring the amount of lectin bound. Lectins used were *Aleuria aurantia* lectin (AAL), *Sambucus nigra* agglutinin (SNA), and *Triticum vulgaris* agglutinin (Wheat germ agglutinin, WGA). Using a calibration curve it was possible to obtain a lectin ratio that was independent of AGP concentration. The lectin ratios were used to quantitate the relative content of carbohydrate structures recognized by each lectin. This allows for comparison of the glycosylation of AGP from different samples, and for studies of changes in AGP glycosylation during the course of a disease. The assay was validated against a lectin ELISA and monosaccharide analysis. The assay was used to follow changes in fucosylation of AGP in patients with severe burns, and to study the effect of cytokines on HepG2 production and glycosylation of AGP.

C1.15

Screening for carbohydrate epitopes in complex glycoconjugate mixtures by fluorescence correlation spectroscopy (FCS)

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Many helminth parasites synthesise characteristic carbohydrates, which can be used as diagnostic markers. To realise this idea one needs two things: a library of epitope specific antibodies and sensitive detection method. Fluorescence Correlation Spectroscopy (FCS) potentially is the method of choice because of its high sensitivity and low sample consumption. FCS exploits the fluorescence fluctuation originating from a small observation volume. The analysis of temporal decay of the autocorrelation function, $G(\tau)$, provides a rate of parameters that characterize the sample, a.o. the diffusion coefficient of the measured species or mixture of species. Use of antibodies as a tag for analysis imposes severe limits: (a) the high molecular weight of antibodies limits the dynamic range of measurement; and (b) every particular antibody should be fluorescently labelled. Here, we propose an experimental solution based on the unique ability of protein A to bind with different affinity to free antibodies and to immune complexes. At least two advantages of this approach are evident: it is not necessary to label the antibody and even the most complex natural glycoconjugates mixture can be considered as a two component model. Possible implications of the approach for epitope screening, diagnosis and characterisation of monoclonal antibodies are discussed, and illustrated with a LewisX – anti- LewisX antibody model.

C1.16

New approaches to the sulphation analysis of glycans

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The sulphation of glycans can confer special properties and may play a key role in recognition and signalling. However sulphated structures generally occur in only a small proportion of the glycans and these are difficult to identify using classical HPLC methodologies.
Oligosaccharides were prepared by keratanase II digestion of keratan sulphates (KS) and then fluorescently labelled. 2-AA labelled material was separated by normal phase HPLC on an amide column and fractions identified using previously calibrated methodologies [1]. 2-AB labelled material was analysed by reverse phase HPLC and by online electrospray mass spectrometry (MS) with microbore HPLC coupled to QTOF MS. This combination of techniques has determined the elution positions of more than 20 characterised structures.
This technique offers higher sensitivity that the traditional methods used. It is now being applied to the investigation of the role of KS in the cornea where it is involved in maintaining tissue transparency and also in the endometrium where embryo attachment appears to be associated with KS-type glycans on mucins [2].

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C1.17

Fluorometric determination of mucin-type glycoproteins by the galactose-oxidase-peroxidase method

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We developed a convenient and specific method for the determination of mucin-type glycoproteins using galactose oxidase and horseradish peroxidase on the basis of the contents of galactose and *N*-acetyl-galactosaminyl residues in glycoproteins. Galactose and galactosaminyl residues released from glycoproteins after hydrolysis were oxidized with galactose oxidase, and subsequently the resultant hydrogen peroxide was determined by a combination of horseradish peroxidase and 3-(*p*-hydroxyphenyl)-propionic acid as a fluorogenic substrate. The contents of galactose/galactosamine residues in N- and O-glycans, as determined by the present method, were in good agreement with those described in the previous reports.
We applied the present method to determine mucin-type glycoproteins indigestive organs. As an application, we observed the release of mucus proteins from rat gastric mucosa by stimulating with misoprostol, a prostaglandin E1 analogue *in vivo*. We also observed the biological role of protease-activated receptor-2 (PAR-2) in secretion of gastric mucus and found that PAR-2 activation triggers secretion of salivary and gastric, but not duodenal mucus.

C1.18

Isolation of lactose-free oligosaccharide fractions from non-human milks

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The problem of removal of lactose is a major bottleneck for the isolation of milk oligosaccharide fractions to be evaluated in biological tests. To solve this problem, different approaches were used. Fractionations were performed on goat, sheep and cow colostra, wheys and milks, and were monitored by HPAEC, paper chromatography, TLC, MALDI mass spectrometry, and NMR spectroscopy. A first approach, based on crystallization of lactose in ethanol/water solutions, has led to removal of only about half of the original lactose and has permitted to isolate by gel chromatography lactose-free acid oligosaccharides. A second approach, based on pre-treatment with β-galactosidase followed by gel chromatography, permitted to isolate an acid oligosaccharide fraction as well a fraction containing most of the neutral oligosaccharides, only minor amounts of these latter remaining with the residual lactose. The HPAEC and MALDI profiles of neutral oligosaccharides were not significantly modified by the enzyme, suggesting that the approach could be used without modification of the compositional/ biological properties of fractions. The HPLC profile of the acid oligosaccharide fractions was substantially unaffected by β-galactosidase A third approach, based on pre-treatment with lactobacilli, also resulted in a satisfactory removal of lactose. (This work has been carried out in the context of the EU project NOFA, FAIR CT 97-3142).

C1.19

Simultaneous quantification of neoglycolipid-coated liposome components by high-performance liquid chromatography with evaporative light scattering detection

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Liposomes composed of dipalmitoylphosphatidylcholine (DPPC), cholesterol, and a neoglycolipid, mannopentaose-conjugated dipalmitoylphosphatidylethanolamine (Man5-DPPE), have been shown to have a strong adjuvant effect in inducing the antigen-specific cellular immunity. Quantification of the components is important for the liposomal quality control. However, the conventional methods to quantify liposomal DPPC and cholesterol involves tedious procedures. Furthermore, there has been no reliable method to quantify neoglycolipids. In this study, a rapid and simple method using a HPLC with an evaporative light scattering detector was developed for simultaneous quantification of the liposome components Man5-DPPE, cholesterol, and DPPC. The chromatographic separation of these components was performed using a trimethylsilane column with an isocratic mobile phase of chloroform-methanol-water after disrupting the liposomes. This HPLC method provided sufficient reproducibility and linearity of calibration curves for quantification of the liposome constituents. In addition, the same chromatographic procedure can also be applied to quantification of various neoglycolipids with different carbohydrate structures. This method will be useful for quality control of neoglycolipid-coated liposomes in studies on drug delivery targeting of carbohydrate receptors.

C1.20

GlycoChip™ - High throughput technology for screening and analysis of protein - glycan interactions

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The GlycoChip™ is the first ever micro-array of complex carbohydrates. Synthesis of the chip glycans are performed *in situ*, through a parallel and spatially addressable sequential chemo-enzymatic process. The range and complexity of the chip carbohydrates are patterned to mimic naturally occurring glycans. The generated glycan binding profiles have extensive applications including: identification of novel carbohydrate binding proteins or carbohydrate processing enzymes, identification of New Chemical Entities (NCE) as potential inhibitors of glycan protein interactions or glycan processing, and analysis of immunogenicity by assaying serum samples for antibody-glycan binding. The Glycochip will permit researchers to efficiently analyze unprecedented numbers of glycan-protein interactions *en masse*.

C1.21

Linear code syntax facilitates the construction of a comprehensive glycomics database and execution of glyco-bioinformatics tools

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The Linear Code™ is a new syntax for representing complex carbohydrates and their associated molecules in a simple linear fashion. Similar to the straightforward nomenclature of DNA and proteins, the novel glycan syntax accounts for all relevant stereochemical and structural configurations in a compact and practical form. The Linear Code is based on a single letter code to represent each monosaccharide. Moreover, it includes a condensed description of the connections between monosaccharides and its modifications. For example, a particular triantennary core-fucosylated bisecting *N*-acetylglucosamine N-linked glycan is written in the following way using condensed IUPAC-IUBMB nomenclature:

Gal(β1-4)GlcNAc(β1-2)[NeuAc(α2-3)Gal(β1-4)GlcNAc(β1-4)]Man(α1-3)-[Gal(β1-4)GlcNAc(β1-2)Man(α1-6)][GlcNAc(β1-4)]Man(β1-4)GlcNAc(β1-4)[Fuc(α1-6)]GlcNAc

Using Linear Code, the same glycan is written as:

GNβ4(Aβ4GNβ2(NNα3Aβ4GNβ4)Mα3)(Aβ4GNβ2Mα6)Mβ4GNβ4(Fα6)GN

The new syntax enables the implementation of bioinformatics tools to execute glycan database searches in Glycominds' Glycomics database and glycan structural comparisons using the Glyder™ algorithm. The Glycomics Database compiles information about glycoconjugate molecules - including their structures, functions, and interactions with other molecules. The Glyder allows the prediction of biological function based on glycan structure. The Glyder measures the level of similarity between two glycans, scores the resemblance, and displays a graphical representation of the result. These tools and database can be accessed at the Glycominds website – www.glycominds.net.

C1.22

Glycosylation site localization of O-glycopeptides by N-terminus derivatization and MALDI-PSD mass spectrometry analysis

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Since the development of electrospray and matrix-assisted laser desorption/ionization modes, mass spectrometry based strategies has considerably grown in the application field to structural biology. Today, mass spectrometry is especially a powerful tool for the characterization of post-translational modifications in proteins. While the interest of mass spectrometry for peptides sequencing is now widely illustrated, the structural analysis of intact O-glycopeptides and the specific O-glycan site localization is often disappointing. Indeed, although the fragmentation pathway depends strongly on the peptide sequence and/or the instrument used, the main cleavage occurs mainly through the loss of the glycan moiety. The O-glycan site localization is consequently circumvented. To overcome this limitation, strategies has been developed based on β-elimination followed by addition of a stable group. However one would expect to limit tedious and sample consuming chemical steps. We will show here how efficient is the simple N-terminus derivatization by a phosphonium group for direct O-glycopeptide full sequencing. Synthetic peptides, derived from mucin MUC5AC motifs, were glycosylated by recombinant GalNAc transferase enzymes and subjected to PSD analysis of their phosphonium derivatives. In contrast to the classical protonated precursor ions, the loss of the glycan moiety is here abolished as the consequence of a likely charge remote fragmentation process. Furthermore, this approach is effective without any purification step before MALDI-PSD analysis.

C2. Carbohydrate-protein and carbohydrate-carbohydrate interactions

C2.1

Investigation of the carbohydrate polymers chitosan and cellulose: Solid phase interaction under conditions of shear deformation

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The polymer blending and modification of polysaccharides under conditions of shear deformation is a promising method because it provides the mixing of components on the molecular level. Under these conditions the reagents were dispersed to molecular size necessary for the chemical reactions in the solid phase. The blends of nature polysaccharides cellulose and chitosan are of great interest since they combine the availability of cellulose and unique properties of chitosan. By chemical structure chitosan, which is deacetyl derivative of chitin, is close to cellulose. This similarity allows producing homogenous blends of the two polysaccharides. To make homogenous blends three different techniques: a two-screw extruder, the Brabender mixer and the Bridgeman anvil were used. The obtained blends were studied with electron microscopy, IR-Fourier spectroscopy, X-ray structural analysis and other methods. It was found that there were no chemical interaction between chitosan and cellulose. IR data showed that between hydroxy- and aminogroups of these polymers the quality different system of hydroxyl bonds is formed that justified the fact of blending of polysaccharides on molecular level.

C2.2

The involvement of carbohydrates in the *in vitro* repair of damage to human respiratory epithelial cell layers

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The airway epithelium provides a primary protective barrier for the human respiratory system. In healthy individuals, if this layer becomes damaged, repair will normally occur. However, in certain diseases such as asthma, repair remains incomplete. Since many biological processes involve glycoconjugates, we postulated that they are also required for the repair of damaged epithelium. We present a model system used to study repair processes *in vitro* using H292 and 16HBE 14o$^-$ epithelial cell layers. After mechanical damage of confluent layers using a plastic pipette tip, the speed of restoration of the layer was measured by image analysis. The role of cell surface glycoconjugates was investigated using lectins. Specific carbohydrate moieties on cells were identified with fluorescent tagged lectins and blocked using lectins added to the culture medium. Two lectins (Wheat Germ Agglutinin and Aleuria aurantia lectin) inhibited the repair of epithelial damage at 48 hours by up to 80% compared to controls, with little effect on cell viability. This inhibitory effect was prevented by pre-incubating the lectin with its specific sugar, demonstrating that inhibition resulted from lectin binding to carbohydrates on the cells. These findings increase our understanding of the role of cell surface carbohydrates in the repair of epithelial damage and afford valuable information which could aid the development of therapies for diseases such as asthma.

C2.3

Synthesis of II^3III^6 bis-sulfated derivatives of isoGM1b as a super ligand for MAG

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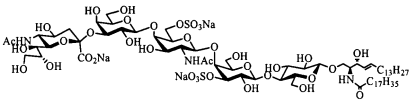
Myelin-associated glycoprotein (MAG), which mediates certain myelin-neuron cell-cell interaction, is a lectin that binds to sialylated glyco-conjugates. In our study to elucidate the ligand structure of MAG at the molecular level, we found that GD1a analogs bearing modified sialic acids at the C-6 of *N*-acetylgalactosamine residue could supported the adhesion of MAG [1]. Based on the results, we designed and synthesized II^3III^6 bis-sulfated derivatives of isoGM1b which possesses isogangliotetraose as a backbone. The glycolipid obtained exhibited much higher activity as a ligand for MAG than GQ1ba, GT1aa, and GD1a.



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C2.4

The role of the O-polysaccharide part of lipopolysaccharides of *Proteus mirabilis* O11, O14, O24, and O29 in complement-mediated killing

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Proteus mirabilis rods are a human urinary tract pathogen. The susceptibility of strains *P. mirabilis* O11, O14, O24 and O29 to bactriocidal action of human complement was examined. The complete structures of the O-polysaccharide part of the lipopolysaccharides (LPSs) *P. mirabilis* O11, O14, O24 and O29 have been established earlier. The main components of the O-polysaccharides studied are hexosamine, hexose and hexuronic acid residues. In addition, the O-polysaccharide part of *P. mirabilis* O14 LPS was substituted by 2-[(R)-1-carboxyethylamino]ethyl phosphate, which determines the serological specificity of the *P. mirabilis* O14a, O14c and O14a, O14b strains. The enhanced ability of complement fixation by *P. mirabilis* O14a, O14c LPS correlates with the susceptibilities of *P. mirabilis* O14a, O14c strain to complement-mediated killing. However, no such correlation was not observed for the other *Proteus* strains and its LPSs studied. The rabbit anti-O sera enhanced the complement fixation by all four LPSs. In conclusion, the differences on O-polysaccharide structures influenced the human complement fixation by LPSs and human complement-mediated killing of *P. mirabilis rods*.

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C2.5

Reinforced frontal affinity chromatography: An effective analytical tool for lectin-carbohydrate interaction

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We established an effective new system for frontal affinity chromatography (FAC), a powerful tool for the analysis of specific interactions between biomolecules [1], adopting an HPLC system equipped with a miniature column and a fluorescence detector. This reinforced FAC allowed us to elucidate detailed binding specificities of lectins very easily, rapidly, and sensitively. A set of accurate dissociation constants (Kd) of dozens of PA-oligosaccharides for one particular lectin can be obtained within a day. This system is unique because it is suited for weak interactions in contrast to almost all other systems. It is therefore advantageous, because lectin-carbohydrate interactions are in general rather weak. A number of both plant and animal lectins have been analyzed. A set of accurate Kd's of oligosaccharides for each lectin composed its profile in terms of the binding property. We found that every lectin has a unique profile, that is, no lectin shared the same profile. Conversely, every oligosaccharide tested also has a unique profile in terms of Kd's for different lectins. Therefore, assignment of unknown oligosaccharides will be possible from its Kd profile.

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C2.6

Binding of a fully sulphated synthetic disaccharide to the carbohydrate-recognition domain of the natural killer cell receptor NKR-P1A observed by NMR spectroscopy

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NKR-P1A is a homodimeric type II transmembrane protein of the C-type lectin-like family found on natural killer (NK) cells and NK-like T cells, and is an activator of cytotoxicity. The natural ligands for this protein are not known [1]. The availability of a well-folded carbohydrate-recognition domain (CRD) [2] allows for exploration by NMR of molecules that it can bind. With transferred NOE's and the saturation transfer difference NMR methods, recently described for screening of mixtures of compounds to detect binding by proteins [3,4], we have observed that a fully sulphated chemically synthesized disaccharide is bound by the NKR-P1A CRD. Although not a natural substance, this molecule may help in elucidating the ligating elements on the natural counter-receptor once identified.

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C2.7

Investigating carbohydrate moiety of glycoproteins using lectins and surface plasmon resonance (SPR) method

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Plant lectins are widely used for characterization of the carbohydrate moiety of glycoproteins; a typical application is ELISA test, where lectins are biotinylated and react with a glycoprotein coated in the microtiter plate wells. SPR method, which is applied in a biosensor BIACORE instrument enables, among others, to measure interaction between native glycoprotein and underivatized lectin. The goal of this work was to establish the range of differences in glycosylation of a glycoprotein; in the preliminary experiments we used human glycophorin A (the major sialoglycoprotein of human erythrocyte membrane) and several lectins: WGA, PHA-E, PNA, APA, SNA, Con A, DSA. In most experiments the lectins were immobilized to CM5 sensor chips (a ligand) and the glycophorin (native and desialylated) samples were in the solutions (an analyte). Obtained results show that the method enables to analyze interaction between glycoproteins and lectins, and the measured reaction reflects known affinity of a lectin to analyzed glycoprotein.

C2.8

Structural basis of the interactions of wheat-germ agglutinin with GlcNAcβ1,6Gal sequence

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Wheat-germ agglutinin (WGA) is a dimeric lectin and the ligand binding sites locate in the interface region between the subunits. In addition to β1,4-linked GlcNAc and sialyloligosaccharide, WGA is also known to interact with GlcNAcβ1,6Gal, which is a characteristic component of branched poly-*N*-acetyllactosaminoglycans in glycoproteins and glycolipids. Our isothermal titration calorimetry analysis showed that GlcNAcβ1,6Gal presented an affinity greater than that of GlcNAcβ1,4GlcNAc for all three major WGA isolectins. In order to obtain the three-dimensional structure information on the interactions of WGA with GlcNAcβ1,6Gal sequence and to compare it to that with GlcNAcβ1,4GlcNAc, we made an X-ray crystallographic analysis of glutaraldehyde-crosslinked WGA isolectin 3 in complex with GlcNAcβ1,6Gal, GlcNAcβ1,4GlcNAc and GlcNAcβ1,6Galβ1,4Glc at 2.4, 2.2 and 2.2 resolution, respectively. The difference electron density maps allocated the ligand molecules in the primary binding sites. The conformation of GlcNAcβ1,6Gal glycosidic linkages varied more extensively compared to that of GlcNAcβ1,4GlcNAc linkage, indicating the large conformational flexibility of the former type of ligand in the interactions with WGA. The difference in the conformation of bound ligands was accompanied by the movement of several side chain groups of amino acid residues in the binding site.

C2.9

Sialic acid-dependent sperm raft-egg raft interactions at fertilization

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Recently, we have isolated the low-density, detergent-insoluble membrane (LD-DIM) fractions or rafts from sea urchin sperm and eggs, and characterized them for the presence of receptor and transducer proteins [1,2]. We hypothesize that the raft of gametic cells functions as an adhesion site as well as in signal transduction during fertilization. In this study, we present the following lines of evidence that sperm raft may interact with egg raft in sialic acid-dependent and independent manners: (i) The sperm LD-DIM bound the 350 kDa sperm binding protein (350 kDa SBP) in the egg LD-DIM. This binding was enhanced by the presence of Ca^{2+}. This Ca^{2+}-enhanced binding disappeared when the egg LD-DIM or 350 kDa SBP was treated with sialidase. No binding was observed when the sperm LD-DIM was treated with sialidase. (ii) The anti-sperm ganglioside antibodies and the recombinant fragments of 350 kDa SBP inhibited the LD-DIM-LD-DIM binding as well as fertilization.

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C2.10

Mannose 6-phosphate receptor like protein (MPR 300) in the invertebrates (Arthropods and Annelidae)

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Mannose 6-phosphate receptor proteins (MPR 300 and MPR 46) play an important role in mediating transport of lysosomal enzymes in mammals. Our earlier studies led to the identification of both the putative receptors among different non-mammalian vertebrates including the earliest vertebrate fish and MPR 300 protein in the invertebrate *Unio*. In order to understand the evolution of the MPR proteins and to further investigate their appearance in other invertebrates, we have isolated proteins from the membrane extracts of Arthropods (*Peneaus indicus*) and Annelidae (*Perionyx excavitus*) that bind to Phosphomannan Sepharose gels and which can be eluted specifically with mannose 6-phosphate. When these eluates were radioiodinated and passed through PM gels, proteins from arthropods and annelidae could be specifically eluted with mannose 6-phosphate. These migrated to the same extent in SDS-PAGE as the goat 300 protein. These radioiodinated proteins failed to bind on *Dictyostelium discoideum* lysosomal enzyme secretions gel and are also not recognised by the anti-goat, anti-frog and anti-Unio MPR 300 antibodies. MPR46 related proteins were not detected in these species.

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C2.11

NMR-spectroscopic and mass-spectrometric studies of the interaction between the Thomsen-Friedenreich antigen (Galβ1-3GalNAcα) and three peptides (minilectins)

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Aromatic stacking and C-H/π-interactions contribute to the energetics and specificity of protein-carbohydrate interactions [1]. With three peptides (P6: ARVSF WRYSSFAPTY, P10: GSWYAWSPLVPSAQI and P30: HGRFILPWWYAFSPS), described as high affinity binding partners [2] for Galβ1,3GalNAcα, we analysed the binding mechanism by 1D and 2D NMR methods, MD simulations and mass spectrometric techniques. Mass-spectrometry showed a rather weak binding in comparison to lectin - sugar interactions and the existence of peptide-peptide complexes. Characteristic signal shifts in the NMR-spectra indicate specific interactions. The medicinal potential of minilectins will be discussed.

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C2.12

Specificity of B-cell Siglecs using sialoside-streptavidin probes

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The Siglecs are a family of 11 members of the Ig superfamily that bind sialyloligosaccharides on glycoproteins and glycolipids [1]. Analysis to date has identified important differences in their specificity. Because the various studies used different assay systems, the combined current information is still fragmentary [1-4]. A sensitive ELISA binding assay was developed using a novel probe consisting of a biotinylated sialoside bound to a streptavidin-alkaline phosphatase conjugate (SAAP). The probes can easily be modified for Siglecs of different specificity by using different synthetic biotinylated sialosides. With this powerful tool, the specificity of Siglecs on human B-cells (Siglecs- 2, 5, 6, 9 and 10) were evaluated in a competitive binding mode using a panel of 20 synthetic sialosides [5]. Significant specificity differences were seen between the Siglecs with respect to the sialic acid (NeuAc/Gc) and the underlying carbohydrate sequence. With the detailed specificity information we are attempting to prepare specific probes for each Siglec that will be useful for *in vitro* cell binding studies using flowcytometry analysis.

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C2.13

Exploring biomolecular carbohydrate epitope recognition – A new approach

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A surface plasmon resonance (SPR) biosensor has been combined with HPLC profiling and fluorescence detection to create a powerful and sensitive method for screening binding of oligosaccharides, present in complex mixtures, to complementary biomolecules. Labelling of the oligosaccharides with 2-aminobenzamide ensures sensitivity in the low femtomole range, whilst the system can differentiate between subtle variations in interaction affinity, and does not affect the biomolecular interaction
In the development of the method, the binding of oligomannose-type structures to concanavalin A was examined, and an affinity rank for the $Man_5GlcNAc_2$ to $Man_9GlcNAc_2$ structures established. In addition, minor fucose-containing constituents in an oligosaccharide mixture, derived from bovine submaxillary gland mucin, were selectively fished out and identified by the fucose-binding lectin from *Lotus tetragonolobus purpureaus*. Further examples demonstrating the utility of the procedure will be included, and possible improvements will be discussed.

C2.14

Chemically synthesised neoglycoconjugates for the prevention of pneumococcal disease

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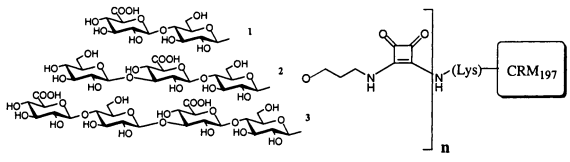
Neoglycoprotein vaccines are in use for the prevention of diseases caused by encapsulated bacteria. A neoglycoconjugate vaccine has recently been introduced against *Streptococcus pneumoniae*, containing polysaccharides coupled to the protein CRM_{197}. In contrast to these complex structures, the use of chemically synthesised oligosaccharides results in neoglycoconjugates with a defined carbohydrate part. These can be used to study the effect of structural details on their immunogenicity, for the development of monoclonal antibodies and as pure antigens in immunoassays.
Synthesised di- (**1**), tri- (**2**), and tetrasaccharide (**3**) fragments related to the capsular polysaccharide (CPS) of *S. pneumoniae* type 3 (see Figure) were coupled to CRM_{197} in varying carbohydrate-protein ratios. The interactions of the conjugates with two CPS-specific antibodies (IgG 3.3 and 3.7) were studied using surface plasmon resonance. The immunogenicity of the conjugates was studied by immunisation experiments in mice.



C2.15

Affinity of proteoglycans and α-elastin in human yellow ligament

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In human yellow ligament, proteoglycan (PG) as the matrix factor and elastic fiber as the fibrous factor are abundantly contained, and it is considered that they are associated with each other, but their interaction has not been clarified. We investigated the affinity between PGs and α-elastin from human yellow ligaments. Two types of small PGs (decorin, biglycan) and a large PG were extracted by ion-exchange chromatography, gel-chromatography and hydrophobic chromatography [1]. α-Elastin was purified by hot alkali methods. The affinity was analyzed by surface plasmon resonance. We immobilized α-elastin on a CM-5 sensorchip and passed a running buffer containing each PG. As a result, it was confirmed that the large PG bound to α-elastin strongly, and the two small PGs did not bind. It is indicated that a large PG gives the physical properties to connective tissues by binding to the elastic fiber and plays important roles in construction of extracellular matrix. We are now investigating the binding site of PGs to α-elastin.

Reference

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C2.16

Interactions of intact human galectin-3 and its C-terminal fragment with oligosaccharides: an electrospray mass spectrometry study

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Galectin-3, the β-galactoside-binding protein plays a central role in a variety of biological functions including cellular recognition and adhesion, regulation of apoptosis and pre-mRNA splicing. We investigated the structure and properties of two galectin-3 proteins using electrospray ionization mass spectrometry (ESI MS), an established method providing direct insights in protein structure and its non-covalent complexes. Intact human galectin-3 (G-3) was expressed in E. coli. The C-terminal fragment thereof (G-3-C) containing the CRD was produced by collagenase VII digestion. Molecular weight of the intact G-3 was determined by nanoESI-QTOF MS to be 26060.2, indicating the lack of the N-terminal methionine, and the ions assigned to adducts with 1-5 lactose moieties. Similar patterns were obtained with the G-3-C preparation. The stability of the lactose adducts and their non-covalent nature was investigated in an ESI-MS approach by de-/renaturation, by increase of the cone voltage and by collision induced dissociation (CID) experiments. Relative affinities for β-galactosides were determined under native conditions.

C2.17

Molecular basis for the carbohydrate specificity of the FimH bacterial adhesin

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The very first step in the colonization of pathogenic *E. coli* of the mammalian urinary tract is the attachment of bacterial adhesins to carbohydrate receptors of the host epithelium. FimH is the mannose-specific adhesin of *E. coli* on the type 1 pilus, which consist of a helical rod composed of repeating FimA subunits and presenting the FimH adhesin at the tip. We here elucidate the interactions of FimH with D-mannopyranoside, the minimal receptor required for specific binding, by X-ray crystallography, in conjunction with a broad mutational analysis of the carbohydrate binding epitope. The mannose-binding pocket is found at the tip of the adhesin, much like the glucamide moeity of C-HEGA in the recently solved structure of FimH in complex with its chaperone, FimC [1]. Asp54 and Glu133 form the bottom of the pocket and are essential for binding as their mutation to similar side chains almost completely abolishes binding. Mutation of the carbohydrate ligand residues Asn46 and Asp140 weakens binding. Other polar interactions involve the N-terminus, the backbone of Asp46 and Asp47, and Asn135. A substantial part of the binding site residues are hydrophobic: Ile13, Ile52, Tyr48 and Phe142. Generally, mutation of any of this abundance of interactions retains the ability to bind mannose coated beads, but all but one mutant lost their ability to hemagglutinate or to bind to a human bladder cell line, 5637 cells.

Reference

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C2.18

A quantitative analysis of carbohydrate-carbohydrate interaction using surface plasmon resonance

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Carbohydrate-carbohydrate interaction plays important roles in cellular recognition and adhesion. The homofilic interaction between Lewis X (Le^x) trisaccharide Characteristic features for this interaction are its extreme low affinity, which is compensated in nature by polyvalence presentation of the ligand, and its dependency of divalent cations. Although the importance of this interaction has been widely accepted the mechanism has not yet been clarified. Lewis X (Le^x) glycosphingolipid is constituted by three moieties: trisaccharides Le^x, lactose and a ceramide. We have studied using SPR the kinetic interaction of Le^x-Le^x and lactose-lactose for mono- and multivalent ligands. The methyl glycosides of each epitopes were used as monovalent ligand and glyconanoparticules were used as polyvalent models. The data obtained enabled us to confirm the importance of the polyvalence and the presence of Ca^{2+}.

The self-aggregation of the sponge, *Microciona prolifera* is also mediated by carbohydrate-carbohydrate interaction. A pyruvylated trisaccharide has been implicated in cellular adhesion. Our SPR study looked at the kinetic of self-recognition of this trisaccharide. Residues that are important in the interaction were prepared and the interaction of their self-recognition was studied. The data obtained enabled us to identify the epitope responsible for self-recognition. These studies allowed us to make some inferences regarding the mechanism of carbohydrate-carbohydrate interaction.

C2.19

Involvement of Galβ1-3GalNAcβ structure in recognition of apoptotic bodies with THP-1 cell line

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The engulfment of apoptotic bodies by phagocytes requires specific recognition system. Some changes in glycosylation could affect the process of apoptotic bodies elimination. Previously we have shown that induction of apoptosis results in loss of most sialic acid- and fucose-containing structures accompanied by an increase in acces-sibility to β-galactose residues on the surface of colon carcinoma cell lines (Glycobiol. **9**, 1337, 1999). Here we studied the ability of PMA-activated THP-1 cell line to engulf the apoptotic bodies generated from human melanoma cell line MELJUSO. Using a set of lectins we have shown that this cell line has the high level of galactose- and sialic acid terminated carbohydrate chains after induction of apoptosis by UV-irradiation. With the help of polyacrylamide probes Sug-PAA-flu we determined that THP-1 cells bound strongly Galβ1-3GalNAcβ ($T_{\beta\beta}$), LacNAcβ, asialoGM1 tetrasaccharide, 3'SL, 6'SL and [Neu5Acα2-8]$_2$ probes that provides participating of galectins and siglecs in engulfment of apoptotic bodies. The phagocytosis was strongly inhibited with $T_{\beta\beta}$-PAA or asialoGM1-PAA but not sialosides. It was confirmed also by inhibition with ECA lectin and absence of inhibition with SNA and MAA. Our results evidence involvement of phagocyte galectins in recognition of apoptotic bodies.

C2.20

Recognition of O-glycan clusters on mucins by lectins

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We previously demonstrated that preferential order and arrangement of GalNAc incorporation into consecutive Thr residues in a portion of the MUC2 tandem repeat were strictly regulated by the specificity of various pp-GalNAc-Ts. The present study was designed to prove O-glycans with different arrangements on mucin core peptides were distinguished by plant lectins. Fluorescein-conjugated peptides (FITC-PTTTPLK) containing GalNAc or Galβ1-3GalNAc residues in a variety of arrangements and combinations were enzymatically prepared. Vicia villosa agglutinin (VVA) and peanut agglutinin (PNA), which preferentially bound GalNAc and Galβ1-3GalNAc residues as saccharide chains, were employed. The fluorescence polarization method was applied to compare affinity of lectins with mucin glycopeptides. Glycopeptides with two GalNAc residues at Thr-2 and Thr-4 strongly bound VVA. The affinity was even stronger than that for peptides with three GalNAc residues. PNA also preferred glycopeptides with two Galβ1-3GalNAc residues at Thr-2 and Thr-4. These results suggest that VVA and PNA recognize arrangement of O-glycans on mucin peptides.

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C2.21

Protein carbohydrate interactions as revealed by SPOT-synthesis technology

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Selective protein-carbohydrate interactions play a central role in many cellular and intercellular events. In an attempt to identify both carbohydrate binding proteins and the structure of the target carbohydrate we use the SPOT-synthesis technology [1]. By using this approach we have covalently coupled various mono- and oligosaccharides via a linker to a cellulose membrane. Subsequently, the membranes were incubated with protein mixtures and protein-carbohydrate interactions were detected by using two approaches: First, the carbohydrate moiety was released from the membrane by UV irradiation and was then analyzed by Matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS). By this technique alterations of the target carbohydrate structure (such as hydrolytic cleavage of intersugar bonds) that might occur during protein-carbohydrate interactions can be detected. Secondly, proteins binding to the immobilized carbohydrates were subjected to tryptic digestion and the resulting peptides were analyzed by MALDI-MS. The peptide masses obtained permit the identification of the interacting protein provided the sequence is known.

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C2.22

NMR and CD study on the interaction between saccharide and β-amyloid peptide

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It is well known that amyloid peptide is a main component of the amyloid plaques found in the brain of patients suffering from Alzheimer's disease. The AD peptide is 39-43 residues long, but its shorter components of 12-28 also shows similar bioactivity. The AD peptide has an characteristic quality of molecular aggregation in aqueous solution, which is reported to be an important initial mechanism of making plaques in the brain. Inhibition of the formation of the aggregation of the amyloid peptides thus emerged as an approach to developing therapeutics for AD. In this paper, we report the CD and NMR spectroscopic analysis of the interaction between 12-28 residues of amyloid peptide and saccharide compound. It was found that the aggregation of peptide was considerably influenced by the interaction between AD peptide and some saccharide compounds.

C2.23

Circulating Cathodic Antigen of *Schistosoma mansoni*: Synthesis of the mono-, di- and trimeric Lewis X epitope and binding studies with anti-carbohydrate monoclonal antibodies

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Adult parasitic schistosomes, parasites which reside in the blood vessels of infected individuals, release high amounts of Circulating Cathodic Antigen (CCA). The immunoreactive part of CCA, which is thought to be important in the modulation of the host's immune response, predominantly consists of repeating Lewis X (Le^x) trisaccharides ($\rightarrow$3Galβ1-4[Fucα1-3]GlcNAcβ1$\rightarrow$). A panel of monoclonal antibodies (Mabs) against CCA and other schistosomal glycoconjugates has been generated from infected mice. To study the structural requirements for the binding of these Mabs to the CCA carbohydrate epitope a Le^x-monomer, -dimer and –trimer were synthesized and conjugated to BSA. The neoglycoconjugates were then used in a binding affinity study with the anti-carbohydrate Mab-panel using a BIAcore. While some Mabs specifically interacted with all Le^x oligosaccharides, others interacted exclusively with dimeric or trimeric Le^x. Several Mabs have been fragmented with the aim to obtain the crystal structure of a Fab-fragment both bound to its matching Le^x oligosaccharide and in its unbound state.

C2.24

Lewis^a and L-Fucα1$\rightarrow$ related ligands in O-glycans as receptors for *Pseudomonas aeruginosa*-II lectin (PA-IIL)

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Pseudomonas aeruginosa -II lectin (PA-IIL) is a rare and important microbial agglutinin that reacts with L-Fuc residues. The affinity of PA-IIL to O-glycans was studied by both the biotin/avidin-mediated microtiter plate lectin-glycan binding assay and inhibition of the agglutinin-glycan interaction. Among 40 glycans tested for binding, PA-IIL reacted well with all human blood group ABH(O) active glycoproteins (gps), but weakly or not at all with their precursor gps as well as nonfucosylated and N-linked gps. Among the oligosaccharides tested by the inhibition assay, the Le^a active pentasaccharide (lacto-N-fucopentaose, LNFP II, Galβ1$\rightarrow$3 [Fucα1$\rightarrow$4]GlcNAcβ1 $\rightarrow$3Galβ1$\rightarrow$4Glc) was the most potent. The decreasing order of PA-IIL affinity toward the oligosaccharides tested was: Le^a pentasaccharide (LNFP-II) $\geq$ sialyl Le^a tetra- and Le^a tri- (Galβ1$\rightarrow$3[Fucα1 $\rightarrow$4]GlcNAc) > methyl αFuc $\geq$ 3'-fucosyllactose (Galβ1$\rightarrow$4[Fucα1 $\rightarrow$3]Glc) > Fuc and Fucα1$\rightarrow$2Gal (human blood group H active disaccharide) > 2'-fucosyllactose, Le^x penta- (LNFP III), Le^b hexa- (LNDFH I) and gluco-analogue of the Le^y hapten (LDFT) > blood group O(H) type I determinant (LNFP I) > Le^x glycotope (Galβ1 $\rightarrow$4[Fucα1$\rightarrow$3]GlcNAc) > sialyl Le^x tri- >> Man >>> Gal, GalNAc, and Glc (inactive).

Reference

Wu AM *et al* (1999) *Glycobiology* **9**:1161-1170

C2.25

Fluorescence polarization to study galectin-ligand interactions

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We have applied fluorescence polarization to measure galectin-ligand interactions and to screen candidate inhibitors. Fluorescein tagged glycosides of LacNAc and Galα1-3LacNAc were synthesized to be used as probes. Binding of probe (0.12–1.2 μM) to different concentrations of unlabeled galectin (0.01–50 μM) in solution was measured in microtiter plate format (96 or 384 well) using a PolarStar instrument (BMG). With increased polarization indicating increased binding of the probe to galectin (about 50 mP for unbound and 200 mP for completely bound probe) highly reproducible binding curves were obtained from which stoichiometry and dissociation constants could be calculated. Measurement at reduced temperature (4 °C) gave a strongly enhanced binding which was useful for detection of low amounts (submicromolar) of galectin and/or galectins with poor affinities for the probe saccharide. In another format the assay was used to measure the relative affinity of unlabeled ligand candidates applied as inhibitors of the interaction of a constant amount of galectin and probe. With the sequencing of the human and other genomes there is an immediate need to screen candidate galectins and other proteins for carbohydrate binding activity. The assay presented here offers an excellent tool for this since it does not require any modification or surface coating of the lectin (protein), is quick and relatively cheap, and can be applied on crude extracts.

C2.26

Self-associated multivalent glycoconjugates strongly inhibiting influenza virus to cell adhesion

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Searching for effective blockers of influenza-virus to cell adhesion we obtained a number of sialoglycopeptides with the following structure: [Sug-linker-$Gly_{n=1-9}$-$NHCH_2$]$_4$C, where Sug- is *N*-acetylneuraminic acid or sialooligosaccharide. Those of them that antennae are built from more than 4 glycine residues are capable to self-association in water solution. The obtained associates are sheet-like structures with planar sizes varied in range of 30-500 nm and certain height (for example, 70 nm in case if n=7). Their interior is two dimensional hexagonal crystal formed by helically folded olygopeptide chains, which are hold together by H-bonds. Both plate surfaces of the associates are covered by carbohydrate moieties making associates water-soluble and providing them with the required antiviral activity. All of the associating sialoglycopeptides demonstrate increased, up to 10^3 times, potency as inhibitors of virus to cell adhesion comparing to monovalent reference, the most active of them block viral adhesion in low micromolar concentration range. It seems that the supra-molecular assemblies interact with virion in the manner like glycopolymers do, i.e. statistically form multiple contacts with viral surface and sterically shield virion from outside.

C2.27

Synergistic interactions of the two classes of ligand, sialyl-Le$^{x/a}$ fuco-oligosaccharides and short sulfo-motifs with operationally distinct binding sites on P- and L-selectin

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The E- L- and P-selectins are adhesion molecules involved in selective leukocyte recruitment in inflammation. 3'-Sialyl- and 3'-sulfo-oligosaccharides of the Le^x and Le^a series are ligands for the selectins, but for high avidity binding of the P- and L-selectins to the glycoprotein counter-receptor, PSGL-1, there is a requirement for the presence of sulfo-tyrosines neighboring a sialyl-Le^x glycan. The P- and L-selectins bind also to other short acidic motifs such as 3-*O*- or 6-*O*-sulfated galactolipids (sulfatides). To examine the functional relationship between the binding sites on the two selectins for these various ligands, we have generated a novel lipid-linked sulfo-tyrosine probe, and used this in binding and inhibition experiments in conjunction with sulfo-glycolipids and neoglycolipids of sialyl- and sulfo-Le^x/Le^a fuco-oligosaccharides. Our results indicate that binding sites for short sulfo-motifs and fuco-oligosaccharides are distinct. The two classes of ligand bind synergistically, and with differing sensitivity to calcium chelation. When presented as mixtures on liposomes, potent inhibitory activities are generated with IC_{50} values in the nanomolar range, and are more than a 100 fold greater than those of the individual ligands. These results have a bearing on the design of therapeutic anti-inflammatory substances and may be relevant to other lectin-type receptors on which dual binding sites, for protein and saccharide, are considered possible.

C2.28

Structural studies of complexes of tetanus toxin Hc fragment and carbohydrate analogues of its receptor ganglioside GT1b

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Tetanus toxin, a member of the *Clostridial* neurotoxins is a Zn metalloprotease consisting of a heavy chain disulfide bridged to a light chain. Prior to exerting its catalytic action the toxin binds via the C-terminal domain of the heavy chain (Hc fragment) at the neuromuscular motor junction, is then internalised and transferred to the central nervous system. The binding of the Hc fragment to the di- and tri- sialogangliosides GD1b and GT1b is a vital stage for the infection. We have characterised by X-ray crystallography complexes of Hc fragment with a heptasaccharide analogue of GT1b and many mono-, di- and trisaccharide subunits of GT1b. The three dimensional structures led to the novel conclusion that there are two binding sites on the toxin, one galactose- and the other sialic acid- specific. Another interesting finding is the observed crosslinking of the protein by the oligosaccharide. Our results can be used for the design of therapeutic agents against tetanus toxin and the structurally and functionally homologous botulinum toxins. In addition, they contribute to an understanding of carbohydrate-protein interactions and the role of carbohydrates in infection.

C2.29

Binding of HDGF and HDGF-related proteins to glycosaminoglycans

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Hepatoma-derived growth factor (HDGF) is the first member identified of a new family of secreted heparin-binding growth factors expressed in different tissues. In a former approach we isolated a new member of the HDGF-related proteins, the HRP-4. Here we examined the quantitative binding of HDGF and the new HRP-4 to different glycosaminoglycans. We found that glucosaminoglycans like heparin or heparan sulfate were bound with high affinity, whereas glycosaminoglycans like chondroitin sulfate were not bound. The specific interaction with these cell surface glycostructures might be important for the so long unclear mode of action of these mitogenic growth factors.

Reference

Izumoto Y *et al* (1997) *Biochem Biophys Res Commun* **238:**26-32

C2.30

Gold glyconanoparticles with polyvalent carbohydrate display for mimicking carbohydrate-carbohydrate interactions

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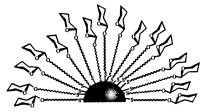
Glyconanoparticles with globular polyvalent carbohydrate display have been obtained by a simple strategy based on gold nanoclusters. Glyconanoparticles prepared with the biological significant lactose disaccharide and Le^x-antigen trisaccharide are stable, water soluble, and can be manipulated as a water soluble biological macromolecules. These nanoparticles offer water soluble 3D-artificial surfaces to study the influence of clustering, sugar concentration, and oligosaccharide presentation in the molecular recognition of carbohydrates in solution. They may be considered as model to mimic glycosphingolipid clustering at the plasma membrane. With these nanoparticles the selective self-recognition of the Le^x-antigen *via* carbohydrate-carbohydrate interactions was studied.



Reference

De la Fuente JM *et al* (2001) *Angew Chem Int Ed*, in press

C2.31

Lysine-based oligosaccharide clusters and complex mannosides specific of human dendritic cells

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Knowing that human blood-monocyte derived dendritic cells (DC) express cell-surface mannose-specific lectins, we prepared various mannose containing glycoconjugates as specific ligands with the aim of developing highly specific synthetic carriers of oligonucleotides and genes. Oligomannose conjugates were prepared after hydrazinolysis of invertase glycopeptides of *Saccharomyces cerevisiae*. The reducing saccharides were converted into glycosynthons, i.e into glyco-aminoacids [1]. Fluorescein tagging was obtained by condensing the free carboxyl group of oligosaccharyl-pyroglutamyl-βalanine and the α-amino group of lysine-*O*-methyl ester previously labelled by fluorescein isothiocyanate on its ε–NH_2. Linear oligolysine containing up to 6 α-D-mannopyranosylphenylthiocarbamyl units have a high affinity for the human mannose receptor [2]. In order to obtain fully biodegradable clusters and to improve both the specificity and the selectivity, dimannosides (Os) were transformed into glycosynthons and those were coupled to oligolysine: $Os_{(n+1)}$-Lys_n-Ala-Cys-NH_2, (where n = 1 up to 5). The thiol group of the cysteine residue was then substituted by reaction with fluorescein iodoacetamide for tagging the conjugate. As shown by flow cytofluorimetry, dimanoside clusters were efficiently taken up by DC and will be used to enhance uptake of glycosylated polyethyleneimine/plasmid complexes by DCs.

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[2] Biessen *et al* (1996) *J Biol Chem* **271**:28024-28030

C2.32

Intracellular trafficking of glycoplexes (plasmid / glycosylated polylysine complexes) in cystic fibrosis airway epithelial cells

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Using glycosylated polylysines as vectors, we have previously shown that the uptake of glycoconjugates containing α-D-mannopyranosyl moieties by immortalized cystic fibrosis airway epithelial cells (Σ-CFTE cells) was much more efficient than that of glycoconjugates containing lactosyl residues, while the gene transfer using lactosylated polylysine was far more efficient than that using mannosylated polylysine [1]. These discrepancies could be linked to a different intracellular trafficking of glycoplexes (plasmid DNA / glycosylated polylysine complexes) depending on the nature of the sugar moieties. We have used Σ-CFTE cells, biotinylated plasmids, fluorescein-labeled lactosylated polylysine, mannosylated polylysine, anti-lamin A/C (for the nucleus) and anti-Lamp1 (for the lysosomes) antibodies, as well as rhodamine-labeled streptavidine. By confocal microscopy, we have shown that more mannosylated than lactosylated complexes were taken up by the cells, (7.2±0.7 and 4.2±0.5 complexes per cell at 30 min, respectively, $p<0.01$). At 8 h, 27% and 42% of the cells had mannosylated and lactosylated complexes present in the nucleus, respectively. At 24 h, more mannosylated complexes were present inside lysosomes, as compared to lactosylated complexes (42±7% and 20±5% of the complexes per cell, respectively). In conclusion, the differential intracellular trafficking *partly* explains the more efficient expression of genes carried by lactosylated complexes, but other factors (such as a preferential dissociation of the lactosylated complexes) are likely to be involved. (Supported by Vaincre la Mucoviscidose).

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[1] Fajac I *et al* (1999) *Hum Gene Ther* **10:**395-406

C2.33

Synthetic carbohydrate probes for studying the carbohydrate-carbohydrate-based interactions in the marine sponge *Microciona prolifera*

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The marine sponge has shown to be an excellent model for investigating recognition and adhesion phenomena in multicellular organisms. A mechanism which has been implied but not yet proven to play a role in sponge cellular aggregation is self-recognition of defined carbohydrate epitopes. Currently, we have developed a system for mimicking the role of carbohydrates in cellular adhesion of the marine sponge *Microciona prolifera*. To this end a spacered sulfated disaccharide epitope, β-D-Glc*p*NAc3*S*-(1→3)-α-L-Fuc*p*-OR, being a fragment of the proteoglycan involved in the Ca^{2+}-dependent aggregation of the cells of the sponge, has been synthesized, and then attached multivalently to BSA using a linker system (diethyl squarate chemistry). Making use of surface plasmon resonance (SPR; CM5 sensor chip), it could be demonstrated that self-recognition is the major force behind the Ca^{2+}-dependent cellular adhesion [1]. In order to study the contribution of the different sites of the disaccharide in the self-recognition process, in a first experiment the contribution of the charge and the *N*-acetyl group of GlcNAc3*S* will be evaluated. To this end two variants of the sulfated disaccharide epitope, β-D-Glc*p*NAc-(1→3)-α-L-Fuc*p*-OR and β-D-Glc*p*3*S*-(1→3)-α-L-Fuc*p*-OR, have been synthesized.

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C3. Cellular adhesion/receptors/transmembrane signaling

C3.1

Minimal requirements for the binding of selectin ligands to a C-type carbohydrate-recognition domain

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The C-type carbohydrate recognition domains of E-selectin and rat serum mannose-binding protein (MBP) have similar structures. Transfer of three lysine residues from E-selectin into MBP creates a chimera that binds the selectin ligand sialyl-Lewis^X. The roles played by Ca^{2+} and by ionic strength in this interaction have been further investigated and extended to the binding of natural selectin glycolipid ligands.
Mannose-binding protein has two Ca^{2+}-binding sites, the second of which is very similar to the single Ca^{2+}-binding site of E-selectin. Intrinsic tryptophan fluorescence studies and solid-phase binding assays demonstrate that binding of sialyl-Lewis^X to the chimera in a selectin-like manner is solely dependent upon the presence of a Ca^{2+}-dependent fucose-binding site, Ca^{2+} site 2, and of an ionic strength-dependent binding site, which consists of the three introduced lysine residues. Binding to glycolipid-coated plates also suggests that the presence of these two subsites is critical to the binding of the non sialyl-Lewis^X-related selectin ligands sulfatides and SGNLs. These results indicate that a Ca^{2+}-dependent fucose-binding site and an ionic strength-dependent binding site are the minimal requirements for the binding of a wide structural range of selectin ligands to a C-type carbohydrate-recognition domain.

C3.2

Gene expression of glycosyltransferases, sulfotransferases and adhesion molecules in human endothelial cells

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The migration of lymphocytes across the high endothelial venules, as well as the recruitment of leukocytes to inflamed tissues occurs as a result of a complex cascade of adhesion and signalling processes in which selectin and selectin ligands mediate the initial tethering and rolling steps.
The selectin ligands require sialylation, fucosylation and sulfation for proper recognition. Thus, the affinity of the leukocyte binding to the endothelial cells may be affected by the state of the glycosylation and sulfation machinery of the latter.
In order to study the genetic regulation of the production of the selectin ligands involved in leukocyte migration we have analyzed differences in gene expression of several glycosyl- and sulfo-transferases as well as adhesion molecules. This assay system used is based on real-time quantitative RT-PCR and has been stablished for HUVECs and endothelial cell lines.

C3.3

Functional interaction between GlcNAc kinase and PKL12, a ser/thr protein kinase involved in cell adhesion control

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PKL12 is the first mammalian member of a new set/thr kinase subfamily not closely related to other protein kinases [1]. It is located at the cytoplasmic site of the Golgi membrane. When overexpressed in adherent cells PKL12 relocated to lamellopodia and filopodium-like structures and promoted cell detachment, indicating a role in cell adhesion control. Based on a yeast two-hybrid analysis an interaction between PKL12 and GlcNAc kinase, an enzyme of the amino sugar metabolism, was revealed. PKL12-GlcNAc kinase interaction was further confirmed both *in vitro* and *in vivo*. GlcNAc kinase was found to be a cytoplasmic protein, but after overexpression of PKL12 both proteins are co-localized in the filopodium-like structures. It was found out that neither GlcNAc kinase was a substrate of PKL12 kinase activity nor GlcNAc kinase influenced PKL12 activity. Furthermore, PKL12 did not influence GlcNAc kinase expression and activity *in vivo*. Therefore functional implications of the molecular interaction between these sugar kinase and a protein kinase remain to be clarified.

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C3.4

Increase in cholesterol level in apoptotic U937 cells treated with dolichyl monophosphate

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Dolichyl monophosphate (dol-P) is an essential carrier lipid in the biosynthesis of *N*-linked glycoprotein. We have reported that dol-P induced apoptosis in human leukemia U937 cells, accompanied by externalization of phosphatidylserine, and various changes in cell physiology. In this study, the consequence of cell membrane perturbation in dol-P-treated U937 cells was investigated. The membrane fluidity reached the maximum within 5 min and remained constant until 40 min. The level of free cholesterol began to increase 1.0 to 1.5 hr after induction and that of total cholesterol almost doubled in 4 hr. By comparing the results with etoposide-treated cells, in which membrane fluidity and free and total cholesterol levels remained unchanged, it is speculated that dol-P first affects the physicochemical characters of membrane and subsequently the composition of lipid, and that these changes are involved in the dol-P-induced apoptosis.

Reference

Fujimoto K *et al* (1999) *FEBS Lett* **446**:113-116

C3.5

Conformation of the macrophage mannose receptor

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The macrophage mannose receptor mediates clearance of pathogenic microorganisms and harmful soluble glycoproteins by recognition of their defining carbohydrate structures. The mannose receptor is the prototype for a family of receptors each having an extracellular region consisting of 8-10 domains related to C-type carbohydrate recognition domains (CRDs), a fibronectin type II repeat and an N-terminal cysteine-rich domain. In the mannose receptor, each of the three types of domain has specific recognition functions. Hydrodynamic analysis and proteolysis experiments performed on the mannose receptor have been used to investigate its conformation. Size and shape parameters derived from sedimentation and diffusion coefficients indicate that the receptor is a monomeric, elongated and asymmetric molecule. Proteolysis experiments indicate the presence of close contacts between several pairs of domains. Hydrodynamic coefficients predicted for modelled receptor conformations are consistent with an extended conformation with close contacts between three pairs of CRDs, and a predicted length for the receptor of 380 Å. The rigid, extended conformation places domains with different functions at distinct positions with respect to the membrane. The relative positions of the CRDs are likely to be important in determining specificity for oligosaccharides of pathogens, whereas projection of the cysteine-rich domain and the fibronectin type II repeat away from the membrane may be important for a proposed role of the receptor in cell adhesion.

C3.6

Syndecan-2 acts as a regulator for actin cytoskeletal organizations in cooperation with or without integrin α5β1 on fibronectin substratum

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Murine Lewis lung carcinoma-derived low (P29) and highly (H11) metastatic clones exhibit different actin cytoskeletal organizations when inoculated on fibronectin (FN) *in vitro*; P29 cells formed stress fibers, whereas H11 cells formed cortex actin. FACS analysis of the FN receptors revealed that P29 cells expressed integrin α5β1 at a similar level to that of H11 cells, but syndecan-2 (SN-2) at a significantly higher level. Using the FN recombinant peptides having Cell-I, Hep-II or a fusion peptide of both domains as substrata, it was demonstrated that (1) the phenotypes of the two clones on FN were reproduced on the fusion peptide, (2) both the clones formed cortex actin on Cell-I domain, and (3) on Hep-II domain, P29 cells formed filopodia but H11 cells did not spread significantly. These results reveal that (1) the extent of spreading in association with cortex actin formation is regulated by signaling through integrin α5β1 alone, (2) the filopodia formation is induced by signaling through SN-2 alone, and (3) the stress fiber formation is induced in cooperation with integrin α5β1 and SN-2. Support for the last mechanism was substantiated by two findings; first, suppression of SN-2 expression of P29 cells with its antisense oligonucleotide resulted in the formation of cortex actin, and secondly over-expression of SN-2 on H11 cells by transfection of SN-2 cDNA caused the formation of stress fibers on FN.

C3.7

Intimins: significance of sugar binding for virulence

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Microbial colonization in and on host organisms is primarily dependent on surface adhesion. In the case of pathogenic microorganisms such surface adhesion factors are usually also important virulence factors. Amongst such adhesion/virulence factors are lectin domain carrying proteins such as the *Yersinia* invasins and the enteropathogenic and enterohemorrhagic *E. coli* (EPEC, EHEC) intimins.

Intimins are outer transmembrane proteins that ultimately, during infection, anchor the bacteria to their own translocated intimin receptor (TIR). The C-terminal end of intimins is at the outside and consists of immunoglobulin-like domains and a C-type lectin domain at the very end. It is well established that the C-type lectin domain is crucial for the manifestation of virulence as seen by an adhesion/effacing lesion phenotype.

At present the sugar binding specificity of intimins is not known. Therefore, we set out to perform structure function studies of different intimins from EPEC and EHEC, and naturally occurring oligosaccharides. To this end, we are using *in silico* molecular modeling combined with *in vitro* adhesion/virulence and binding studies. Here, we present our progress in unraveling the mysteries of intimin-sugar binding with special emphasis on its implication for EPEC and EHEC virulence.

C3.8

Constitutive and developmentally regulated glycosylations of CAMs mediate sequential steps in synaptic targeting

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Leech neurons are labeled with both constitutive and developmentally regulated glycosylations of their cell adhesion molecules leechCAM and Tractin, orthologs of NCAM and L1. Their constitutive mannose epitope, recognized by mAb Lan3-2, mediates branching as neurons reach their target. Subsequently, developmentally regulated galactose epitopes divide sensory neurons into different subsets. These galactose epitopes now inhibit branching and mediate synapse formation. In Triton X-114 solubility assays, leechCAM and Tractin behave as peripheral membrane proteins. Ultrastructurally, the mannose and galactose epitopes are detected on adjacent glial cells, consistent with shedding of glycoprotein. Culturing embryos in the presence of serine protease inhibitor CMK inhibits the proteolytic cleavage of 260 kD Tractin to its final 130 kD size. As determined by video imaging, exposing sensory afferents to CMK results in the elongation of higher order branches. With less shedding, the higher concentration of mannose epitope may allow for increased adhesion resulting in greater branch length. The chemical structure of N-linked carbohydrate chain bearing the mannose epitope is under investigation using NMR spectroscopy and FAB-MS, GC-MS, and MALDI-TOFMS.

C3.9

Dolichyl monophosphate induces mitochondrial disruption and apoptosis in U937 cells

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Dolichyl monophosphate (Dol-P) is involved in the attachment of carbohydrate chains to proteins in the formation of N-linked glycoprotein. We found that this compound induces apoptosis in human monoblatic leukemia U937 cells. In Dol-P treated U937 cells, increase of membrane fluidity, increase of caspase-3-like protease activity and DNA ladder formation were observed successively. In this study, we examined mitochondrial morphological changes by electron microscope and mitochondrial transmenbrane potential ($\Delta\Psi$m) by JC-1. Mitochondrial disruption and reduction of $\Delta\Psi$m were observed immediately after treatment of Dol-P. Subsequently, cytochrome c was released from mitochondria and diffused in cytoplasm. Apoptosis-inducing factor was translocated around nuclei. These results indicate that mitochondrial disruption is important for triggering of apoptosis.

Reference

Yasugi E *et al* (2000) *FEBS Lett* **480**:197-200

C3.10

Polysialic acid and NCAM modulate a phosphotyrosine signal implicated in cell growth and neuronal differentiation

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Polysialic acid (PSA) on the neural cell adhesion molecule NCAM modulates cell interactions. Asking, whether PSA modifies NCAM-mediated second messenger pathways, we studied PSA- and NCAM-dependent changes in the phosphotyrosine pattern of neuroblastoma cells expressing high levels of PSA-NCAM [1,2]. Incubation with NCAM or a ligand of NCAM as well as enzymatic removal of PSA induced a transient increase in the intensity of a 35 kDa band. A marked increase of this band was also observed in high density cultures with a diminished polysialylation of NCAM and a reduced growth rate. Thus, the 35 kDa phosphotyrosine signal may be involved in NCAM-dependent growth inhibition. During neuronal differentiation, the 35 kDa band vanished along with cdk1 and cdk2 expression. However, it co-migrates with none of the cdks analyzed by immunoblotting. In conclusion, our data identify a new element of a NCAM-mediated signal cascade and provide the first evidence that PSA modulates NCAM-signaling involved in cell growth and differentiation. (Supported by DFG grant Hi 678/ 2-1).

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C3.11

Endothelial cells endogenous lectins are distinctive signals for adhesion/invasion by circulating cells

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Molecules and cells entry from blood to an organ is controlled by endothelial cells (EC) lining the vessels. This highly specific selection depends upon EC surface adhesion molecules and EC lectins are involved in this cascade. They differ as related to tissue localisation, the type of vessel EC belong to and biological processes.To identify which molecules make them distinct, endothelial cells from various tissue origins have been established as lines which kept their phenotype and recognition specificity towards circulating normal and tumor cells. This cellular model was used to approach the molecular specificity of EC-mediated selection processes. Distinct biological properties were kept and illustrated by endogenous lectins expression in resting, as compared to activated conditions. EC organospecificity of endogenous lectins was demonstrated by differential display of gene expression in endothelial cells from distinct tissue origins, in resting and activating conditions, i.e. through cytokine and chemokine receptors. Because EC endogenous lectins are selectively modulated in the above described conditions, they may help define glycosylated molecules able to interfere with EC adhesion capacity in pathological processes.

C3.12

Cloning and characterization of murine Siglecs

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Siglecs are type 1 membrane proteins which recognize a sialic acid containing carbohydrate group as a ligand. Siglecs are associated with several biological processes like hemopoiesis, neuronal development and immunity [1,2]. By PCR-based methods, with primers based on human Siglec-9, we have cloned a cDNA from murine spleen encoding a member of the Siglec family. The Siglec exhibits a high degree of sequence similarity to both human Siglec-7 and 9 as also recently reported by others [3]. A second cDNA homologous to human Siglec-10 was obtained by PCR from murine spleen mRNA using primers obtained from murine EST and murine genomic sequences [4]. For the evaluation of the binding specificity of these murine Siglecs, sialoside probes have been synthesized using a chemo-enzymatic approach [5]. By the use of these probes and CHO cells stably expressing these Siglecs, we have started to characterize their detailed specificities. This information will be used to develop sialyloligosaccharide probes for each murine Siglec to be used as tools in the investigation of their biological roles.

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C3.13

GlcNAc-T V expression alters cell-fibronectin adhesion and migration via beta 1 integrin N-linked oligosaccharide branching

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GlcNAc-T V (Mgat5) activity is up-regulated after oncogenic transformation and in many human tumors by activation of the ras-raf-ets pathway and enhanced transcription of the enzyme. This effect causes increased β(1,6) branching of the complex N-linked oligosaccharides of a restricted set of glycoproteins. To document effects of β(1,6) branching on cell-matrix interactions, NIH3T3, HT1080 human fibrosarcoma, and human dermal fibroblasts were infected with retroviral constructs encoding GlcNAc-T V under the control of a tet-off inducible promoter. Increased enzyme activity in each cell type caused decreased adhesion to fibronectin, and this adhesion was inhibited by antibodies specific for the alpha 5 and beta 1 integrin subunits. Moreover, migration toward fibronectin (haptotaxis) was significantly stimulated in cells expressing induced GlcNAc-T V levels. Several experimental approaches showed unaltered levels of cell surface alpha 5 beta1 after induction of GlcNAc-T V activity, suggesting changes in β(1,6) branching caused altered integrin binding affinity for fibronectin. Surprisingly, alpha 5 beta 1 purified from HT1080 cells before and after induction of GlcNAc-T V shows no expression of β(1,6) branching on the alpha 5 subunit. Beta 1 shows a basal level of branching that is greatly increased after induction, suggesting that the effects of increased GlcNAc-T V expression on decreased integrin adhesion are mediated via the beta subunit and stimulate cell-matrix migration.

C3.14

Expression of O-glycans on activated human T lymphocytes from cord blood

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Mucin-like molecules in the membrane of lymphocytes has been shown to play a relevant role in cell communication and activation. The GalNAc specific lectin *Amaranthus leucocarpus* (ALL) induces immunosupression and recognizes murine and human lymphocytes in the last stages of maturation. In this work we report the association of expression of O-glycans that bind ALL with CD45RA+ phenotype in CD4+ T lymphocytes from cord blood. Flow cytometry analysis indicated ALL recognizes 5-8% of non-activated lymphocytes, whereas, ConA stimulation increased this receptor 28% at 36 h and 46% at 48 h after activation the ALL+ T cells, in all cases the ALL-R is a glycoprotein of 70 kDa. The expression of the ALL receptor correlates with the expression of CD69 an early activation marker. Diminution of the ALL receptor is observed with the increase in the KI67 expression. The receptor has been purified by affinity chromatography and showed no homology with other known T-cell markers. Our results indicated that ALL recognizes specific O-glycosidically linked glycans which seems to participate in early activation of naïve human T cells. (Financed in part by CONACyT and DGAPA-UNAM, Mexico).

C3.15

Differential expression of a 70 kDa O-glycosylprotein on T cells: a marker of murine naïve T cells

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Among the various cell surface glycans, the biological function of O-glycans, containing basically GalNAc-Ser/Thr, is not well understood, but several reports suggest that these molecules are involved in the modulation of cell-cell interactions. In this work we purified an O-glycosylated protein which is recognized specifically by the *Amaranthus leucocarpus* lectin, and we determined its expression pattern on T lymphocytes from murine lymphoid organs. The purified receptor is a glycoprotein of 70 kDa (ALLr), and it was demonstrated to be present in 95% of $CD4^-8^+$ and $CD4^+8^+$ thymocytes, and in 56% of $CD4^+8^-$ thymocytes. 90% of peripheral blood, 80% of spleen, and 95% of lymph node $CD8^+$ T cells were ALL^+; and the $CD4^+$ ALL^+ T cells were 12%, 11%, and 9%, respectively. Most of the ALL^+ T cells showed a $CD62L^{hi}$ $CD45RB^{hi}CD44^{lo}$ phenotype, indicating features of naive cells. CD19+ B cells from bone marrow but not peripheral B cells were recognized by ALL. Mitogenic activation increased maximal ALL binding to $CD4^+$ T lymphoblasts 24 h after stimulation, but not to $CD8^+$ T lymphoblasts. Our results suggest that ALLr is expressed on quiescent naive $CD8^+$ T cells, as well as during early activation stages of $CD4^+$ T cells. (Financed in part by CONACyT and PAPIIT-UNAM, México).

C3.16

The $\alpha_3\beta_1$ integrin glycans from HCV cell line

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Integrins are glycoproteins that exist as heterodimers. There is some evidence that cell adhesion regulated by integrins is modulated by glycosylation. Alteration of the integrin glycosylation have often been demonstrated in transformed cells.
Here, the characterisation of carbohydrate moieties of $\alpha_3\beta_1$integrin from cultured human non-malignant transitional epithelial cells of ureter – HCV 29 was carried out. $\alpha_3\beta_1$ integrin from the HCV 29 cell line was purified on the peptide GD-6 column. Using MALDI MS the glycan composition of both subunits was determined after SDS-PAGE separation under non-reducing conditions, blotting on PVDF and PNG-ase F digestion. Both subunits were almost uniformly glycosylated possessing complex biantennary structures as well as *N*-acetyllactosamine repeats. Most of the glycans were core and branch fucosylated. Additionally, the α subunit contained high mannose type glycans with core fucose.

C3.17

Identification of glycoprotein IIb-IIIa (integrin αIIbβ3) as the main galectin-3 ligand on the platelet surface

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Galectin-3 is a β-galactoside-binding protein implicated in diverse biological processes involved in cellular interactions. The biological functions of galectin-3 remain to be further elucidated. The identification of galectin-3 native ligands will facilitate the understanding of the mechanism of galectin-3 functions. In order to identify the ligands, the human platelet lysate was affinity-purified on immobilized recombinant human galectin-3 and lactose eluted ligands were analyzed by MALDI-TOF mass spectrometry. The main isolated protein was glycoprotein IIb-IIIa (CD41a antigen). Other components were filamin and thrombospondin, possibly associated with glycoprotein IIb-IIIa, and KIAA0803. Flow cytometry demonstrated that 92% of the population gated by anti-CD41a was galectin-3 positive. Galectin-3 binding to the ligands on the platelet surface can be inhibited by lactose in a dose dependent way. The glycoprotein IIb-IIIa complex is the most abundant platelet adhesion receptor, and mediates platelet aggregation, firm adhesion, and spreading. Galectin-3 did not induce aggregation and also did not inhibit the aggregation induced by ADP.

C3.18

Novel O-linked oligosaccharides that function as L-selectin ligands

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It is critical for lymphocyte homing that L-selectin binds to L-selectin ligands, sulfated sialyl Lewis x present on high endothelial venules (HEV). Recently cloned L-selectin ligand sulfotransferase (LSST) formed core2-based L-selectin ligand functional in rolling assays [1,2]. Analysis of core2GnT-I gene knockout mice indicates that lymphocyte homing and expression of MECA-79 antigen persist after the gene inactivation [3]. Structural analysis of L-selectin ligands in HEV of the knockout mice demonstrated that the major oligosaccharides remaining are based on extended core1 structure such as NeuAcα2-3Galβ1-4[(Fucα1-3)sulfo-6GlcNAc]β1-3Galβ1-3GalNAcα1-Ser/Thr. Human LSST together with the novel β1,3GnT, which forms extended core1 structure, formed Galβ1-4(sulfo-6GlcNAc)β1-3Galβ1-3GalNAcα1-R on CHO cells, resulting in MECA-79 epitope expression. 6-Sulfo sialyl Lewis x expressed on extended core1 serves as L-selectin ligand under shear forces. Moreover, twin 6-sulfo sialyl Lewis x on biantennary O-glycan consisting of extended core1 and core2 served as better L-selectin ligand. (Supported by CA71932 and CA48737).

References

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C3.19

Sialyl-oligosaccharides inhibit the adhesion of *Escherichia coli* and *Salmonella fyris* to Caco-2 cells

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Background: It is well-known that viruses, bacteria or toxins develop their pathogenic effect against a cell only through the adhesion to receptors located on the epithelial surface. Oligosaccharides of human milk, thanks to their particular structure, are able to compete with receptors in binding pathogenic agents and hindering their adhesion as well as the subsequent process.
Method: We carried out several experiments using differentiated Caco-2 cells (line ATCC HTB37) to study the effect of some sialyl-oligosaccharides [synthesized 3'sialyl-lactose (s3'SL), a mixture of 3' and 6'sialyl-lactose of human milk (3'-6'SL), a mixture of acidic sialyl-oligosaccharides of human milk (SO), a mixture of sialyl-oligo-saccharides of goat milk (gSO)], and lactose (L) on the adhesion of *Escherichia coli* O:119 (EPEC) and *Salmonella fyris* B8132.
Results: Inhibition of the adhesion of E. coli: s3'SL = 26%; 3'-6'SL = 13%; SO = 34%; gSO = no inhibition; lactose: no inhibition. *Inhibition of the adhesion of* S. fyris: s3'SL = no inhibition; 3'-6'SL = 16%; SO = 16%; gSO = 24%; lactose: no inhibition.
Conclusions: Our results demonstrate that some sialyl-oligosaccharides, thanks to a selective receptor-like mechanism, inhibit the adhesion of *E. coli* and other ones inhibit the adhesion of *S. fyris* to cultured intestinal cells. These results prove that human milk plays an important role in protecting the newborn against infections.

Reference

Coppa GV *et al*, In: RAA Muzzarelli, chitosan per os: from dietary supplement to drug carrier. Atec Ed (2000) 313-330

C3.20

Differential effects of *H*-type α(1,2)-fucosyltransferase on E- and P-selectin cell binding

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The sialyl-Lewis epitopes (sLe^x and its isomer sLe^a) are abundantly expressed on human carcinomas and have been shown to play an important role in tumor metastasis. We have recently reported that when the *H*-type α(1,2)-fucosyltransferase (HT) is expressed in α(1,3)-fucosyltransferase FucTVII-expressing cells, the sLe^x precursor is preferentially converted to the histo-blood group H structure which cannot be further fucosylated by FucT-VII, leading to a dramatic decrease of sLe^x expression and E-selectin adhesion [1]. In the present study, we engeneered a recombinant HT fused either to the Enhanced Green Fluorescent Protein (HT-EGFP) or the red fluorescent protein from the coral Discosoma (HT-DsRed), and generated stable tumor cell transfectants to examine the effect of HT overexpression on the synthesis of sialyl-Lewis epitopes as well as E- and P-selectin interaction. To analyse E-selectin binding, we used 3 different tumor cell lines including the hepatocarcinoma HepG2, the colonic HT29 and the pancreatic BxPC3 cells. To analyse the effect of HT transfection on P-selectin binding, we used CHO cells expressing the required molecules FucTVII, the P-Selectin Glycoprotein Ligand-1 (PSGL-1) and the β(1,6)-*N*-acetylglucosaminyltransferase (C2GnT; F7.P1.C2 cells). Our data show that the fluorescence level of HT-EGFP and HT-DsRed expression is proportional to the extent of sLe^x and sLe^a inhibition. However, although a complete inhibition of sialyl-Lewis epitopes can be reached in all the 3 tumor cell lines tested, only a moderate effect on E-selectin adhesion can be observed. P-Selectin binding to F7.P1.C2 cells however, can be completely abolished following HT-EGFP or HT-DsRed transfection.

Reference

Zerfaoui et al (2000) *Eur J Biochem* **267** :53-60

C3.21

Detection of a glycoprotein ligand for Siglec-6 on placental cells

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The recent discoveries of Siglecs (sialic acid-binding immunoglobulin-like lectins)-5-10 revealed a sub-family of molecules with a close homology to Siglec-3 (CD33). One of these, Siglec-6, is expressed on placental trophoblasts and B cells and recognizes the sialyl-Tn epitope. This is the first Siglec for which a protein ligand has also been found, i.e. leptin. To explore the biological functions of Siglec-6, we have set out to find its natural (glyco)-conjugate ligand(s). Flow cytometry analysis showed virtually no binding of Siglec-6-Fc (the 3 extracellular domains of human Siglec-6 fused with the Fc part of human IgG) to cells of hematopoietic origin (Daudi, Jurkat, HL60, U937, THP-1, HEL). In contrast, binding was observed to cells of placental origin (JAR and BeWo) and to cells of endometrial origin (SNG-M). For most Siglecs a specific arginine residue in the first domain is critical for sialic acid recognition. A Siglec-6-Fc in which this arginine was mutated to an alanine, showed considerably reduced binding to JAR, BeWo and SNG-M cells, suggesting that the interaction is at least partly sialic acid dependent. Treatment of JAR and BeWo cells with trypsin abolishes binding by Siglec-6-Fc, indicating that the ligand(s) may be sialylated glycans presented on cell surface glycoprotein(s). Immunohisto-chemistry using Siglec-6-Fc revealed the presence of ligands in human placenta mainly on decidual cells (maternal origin). The presence of Siglec-6 and its ligand in placenta suggests a role for this Siglec in pregnancy. Maternal sections of placentas (rich in decidual cells) are being studied to further characterize and purify this/these natural ligand(s) for human Siglec-6.

C3.22

Modulation of matrixmetalloproteinase production in monocyte-macrophage by matrix protein substratum

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Blood monocytes during their transendothelial migration encounter with subendothelial matrix and differentiate into macrophages. As part of our investigations to study the influence of extracellular matrix on monocyte-macrophage differentiation, an *in vitro* model system with isolated human PBMC maintained in culture on different matrix proteins was employed. Expression of the MMPs was studied as a macrophage specific marker activity. As the duration of the culture increased there was a progressive increase in the expression of MMP 2 and MMP 9 and significantly higher rate of production of MMPs was found in cells maintained on Fn and col I than col IV. Anti-integrin antibodies and blocking peptides reversed Fn dependent up-regulation of MMPs suggesting that matrix effect was mediated through integrin receptors. Blocking of Fn dependent effect on MMP production by genstein indicated phosphorylation dependent downstream signalling. Blotting with phosphotyrosine antibodies indicated the transient phosphorylation of two intracellular proteins in a substratum dependent manner. These results indicate that in monocyte-macrophage, matrix protein substratum modulates production of MMPs which play an important role in matrix remodelling and further suggests that apart from diffusible factors, monocyte-macrophage differentiation is influenced by extracellular matrix.

C3.23

C-mannosylation and O-fucoslyation of thrombospondin type 1 repeats

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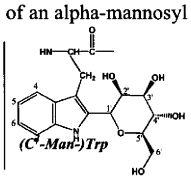
Protein C-mannosylation involves the attachment of an alpha-mannosyl residue to the side chain of tryptophan:



The reaction is catalysed by a microsomal transferase that is found in a wide range of organisms and recognizes the motif Trp-x-x-Trp. This sequence occurs in most thrombospondin type 1 repeats (TSRs), a module present in a large family of proteins with diverse functions. Peptides from thrombospondin-1, properdin, F-spondin and complement proteins C6-9 were examined by mass spectrometry and Edman degradation. The 24 TSRs found in these proteins were all C-mannosylated; containing a total of 46 (C^2-Man-)Trp residues. In the course of these studies it was found that the TSRs in TSP-1 undergo a second unusual glycosylation. Ser and Thr residues in three CSXS/TCG motifs carry the O-linked disaccharide Glc-Fuc-O-Ser/Thr. This is the first protein in which such a disaccharide has been identified. Both C- and O-glycosylations take place on residues that have been implicated in the interaction of TSP-1 with glycosaminoglycans or other cellular receptors. These results form the basis for functional studies of these modifications.

C3.24

The role of N- and O-glycosylation in sperm-egg recognition and binding at fertilization

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There is evidence that, in both invertebrates and vertebrates, sperm-egg recognition and binding at fertilization is an event mediated by lectin-like molecules on the sperm plasma membrane and a sugar ligand on the egg extracellular coat. In the mollusk bivalve *Unio elongatulus* the ligand is gp273 and it exerts its function through O-glycans. Five different classes of glycans have been characterized by chromatographic methods and their biological activity determined by a solid phase binding assay. From these studies emerged the involvement of terminal fucose residues and sulfate groups in the interaction. The primary structure of the gp273 N-linked chains has also been identified and their lack of any function in the interaction clearly determined. The only two structures found were $Glc_1Man_9GlcNAc_2$ and $Man_9GlcNAc_2$ in a 3:7 ratio. The different roles played by N- and O-linked glycans in fertilization is discussed in the light of the evolutionary development of functions associated with oligosaccharides.

C4. Developmental glycobiology

C4.1

Stage- and tissue-specific expression of *N*-acetylglucosamine β-1,4-galactosyltransferase AKI in the embryonic epidermis

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Changes in oligosaccharide structures of glycoconjugates have been observed and are postulated to have key roles in embryonic development and differentiation. We wanted to know whether, or not, AKI is expressed and modifies glycosylation patterns in mouse embryos. Northern blotting, Western blotting, *in situ* hybridization, GalT activity assay, AKI binding assay, radioisotope labeling of AKI substrate glycoproteins were used as methods. AKI showed different expression patterns in time and space, and different enzymatic activity from other known family members. Epidermis of mouse embryo included high level of AKI activities, which transferred galactose to endogenous glycoprotein (MW 130 kDa) (GP130). The maximum of the activity was for 13.5 days-post-coitum embryos. Specific antibody against AKI inhibited 81% of *N*-acetylglucosamine β-1,4-galactosyltransferase activities, which indicates that AKI represents the major part of embryonic epidermis enzymes. AKI shows 2.2 times higher galactosyltransferase activity towards galactose-acceptor glucose with α-lactalbumin than towards *N*-acetylglucosamine without α-lactalbumin. AKI is also expressed in mouse melanoma and leukemia cell lines and in human basal cell carcinoma specimens. In conclusion, the GP130 galactose acceptor once galactosylated by AKI may be directly involved in epidermal differentiation and oncogenesis.

Reference

Uehara K and Muramatsu T (1997) *Eur J Biochem* **244**:706-712

C4.2

Expression of chondroitin 6-sulfate in neuroepithelial boundaries in mouse embryos

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Rhombomeres are temporary repetitive structures important in patterning subsequent hindbrain development during early mouse embryogenesis. As chondroitin 6-sulfate (C6S) was the major chondroitin sulfate (CS) isotype found in hindbrain, we hypothesized that C6S may be involved in hindbrain development. To address this, *in situ* hybridization and immunohistochemistry were carried out on both sectioned and whole-mount mouse embryos from stages 8.5 to 11.5 dpc. We chose to use the monoclonal antibody CS-56, which we showed to be immunoreactive to C6S epitope in proteoglycans. In both tissue sections and whole-mount embryos, immunopositivity was located in the neuroepithelial boundaries. The signals were found stronger in E 8.5 and E 9.5 embryos than in later stages. As C6S is most likely a product of chondroitin 6-sulfotransferase (C6ST)-catalyzed reaction, we reasoned that upregulation of C6ST gene expression took place in the boundary regions. A riboprobe specific for the C6ST gene was synthesized and used for *in situ* hybridization. Signals were found between rhombomeric boundaries in E 8.5 and E 9.5 mouse embryos but not in E 10.5 and E 11.5 embryos. These results imply that C6S may be involved in the formation or maintenance of the boundaries. Further work will be performed to identify signals that trigger the *in situ* positive cells to change in C6ST expression and to test the consequence of this change to the formation of rhombomeric boundaries.

C4.3

The role of complex N-glycans in metazoan development

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There is an evolutionary boundary between unicellular and multicellular eukaryotes in that only the latter can make complex N-glycans. Complex N-glycan synthesis is initiated by UDP-GlcNAc:α-3-D-Man β-1,2-GlcNAc-transferase I (GnTI). Mouse embryos with a null mutation in this gene do not survive beyond 10.5 days post-fertilization. We have cloned three *Caenorhabditis elegans* genes homologous to mammalian GnTI (designated *gly-12*, *gly-13*, and *gly-14*). Expression in transgenic worms showed that *gly-12* and *gly-14*, but not *gly-13*, encode active GnTI when assayed with Manα1,6(Manα1,3)-Manβ1-octyl (Man_3-octyl). Homozygous *C. elegans* single null mutants for the *gly-12* and *gly-14* genes and the double null mutant [*gly-12;gly-14*] display wild type phenotypes. The double mutant shows no detect-able GnTI activity with Man_3-octyl but the worms synthesize complex N-glycans due to *gly-13* which encodes an active GnTI when assayed with the physiological $Man_5GlcNAc_2$-R substrate. The major GnTI activity in the worm is due to *gly-13*. The homozygous *gly-13* null mutation is associated with lethality at an early larval stage. Complex N-glycans appear to be essential for normal development in humans, mice and worms.

C4.4

A survey of putative UDP-*N*-acetylgalactosamine: polypeptide *N*-acetylgalactosaminyltransferase genes in the genome of *Drosophila melanogaster*

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A survey of the fruitfly genome identified 15 homologs that are putative GalNAc-transferase genes. The coding regions were assembled for 12 genes predicted to encode type II transmembrane proteins. These contained distinct domains that were similar to mammalian GalNAc-transferases. Phylogenetic analysis of the putative catalytic domains of *D. melanogaster*, *C. elegans*, and mammalian homologs of GalNAc-transferase gene families revealed conservation of several subfamilies of putative orthologous genes among one or more members in each species. One distinct subfamily was represented by the *l(2)35Aa* gene of *Drosophila.* The *l(2)35Aa* gene was previously shown to be essential for embryonal development [1]. The *l(2)35Aa* gene was cloned, expressed and shown to encode a functional GalNAc-transferase designated *d*GalNAc-T1. *d*GalNAc-T1 exhibits higher expression levels in female adults especially in ovary, and *in situ* hybridization studies demonstrated expression in all stages of egg development.

Reference

[1] Flores C and Engels W (1999) *Proc Natl Acad Sci USA* **96:**2964-2969

C4.5

Systematic analysis of genes involved in glycome formation of the nematode *Caenorhabditis elegans*

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The nematode *C. elegans* could be the most potent animal model for studying functions of glycoconjugates in development and morphogenesis. To dissect their functions in cell, developmental and morphogenetic pathways, we can now use DNA sequence data from the genome sequence project and a wealth of cell-lineage/mutant information. Moreover, functions of specific genes can be assessed by RNA mediated interference (RNAi) or by isolating deletion mutants of the genes. In this study, we report results of systematic RNAi of the genes involved in carbohydrate metabolism, carbohydrate recognition and modification. For instance, inhibition of a galectin gene or a putative acetyl-CoA transporter gene (AT-1) possibly involved in *O*-acetylation of glycoconjugates induced embryonic cell death. An isolated AT-1 weak allele and other mutants were analyzed with 4-dimensional microscopy and expression patterns of various genes were analyzed with real time PCR and other techniques.

C4.6

Expression of fucosyltransferases during chick embryogenesis

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The L5 epitope has been identified as an Le^x-like fucosylated glycan and is implicated in neural induction in the chick embryo which represents one of the very early developmental steps of the nervous system [1]. In order to investigate whether a fucosyltransferase (FUT) is upregulated to generate L5, we analysed the expression patterns of FUTs by whole mount in situ hybridization and RT-PCR. Remarkably, we observed fairly similar expression patterns for FUT4, FUT9 and another FUT which was cloned from a genomic chick library and identified as an orthologous homologue of the ancestor gene of the human and chimpanzee FUT3, FUT5 and FUT6. The expression patterns of the FUTs and L5 clearly overlapped, but they were not identical. Further studies will show whether one or more FUTs can be upregulated by Hepatocyte Growth Factor/Scatter Factor in a way as has been shown for L5 [1].

Reference

[1] Streit A *et al* (1997) *Development* **124:**1191-1202

C4.7

Structural analysis of sialylated N-linked oligosaccharides in murine immortalized motor neuron-like cells (NSC-34)

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To analyze the property of motor neurons, the immortalized motor neuron-like cells NSC-34 were developed by fusing neuroblastoma N18TG2 with motor neuron-enriched embryonic day 12-14 spinal cord cells. The function of N-linked oligosaccharides has been known little in motor neurons. Therefore, at first, we studied comparative structural analysis of N-linked oligosaccharides between N18TG2 and NSC-34 to elucidate the function of N-linked oligosaccharides in motor neurons. N-linked oligosaccharides were released from two cell lines by hydrazinolysis. The reducing ends of oligosaccharides were labeled with *p*-aminobenzoic octyl ester (ABOE). ABOE-labeled oligosaccharides were subjected to anion exchange HPLC and neutral fraction (N fraction) and acidic fraction (A fraction) were separately pooled. Acidic fractions were digested with neuraminidase. Neutralized oligosaccharides (AN fractions) were subjected to reverse-phase HPLC. As a result, we found several differences in elution profiles between oligosaccharides derived from N18TG2 and NSC-34. It is suggested that oligosaccharides increased in NSC-34 compared with N18TG2 modulate cell migration and cell adhesion in motor neuron specific neurite outgrowth. It is very important to analyze how these oligosaccharides play a role in NSC-34.

C4.8

Differential nuclear protein expression and glycosylation in *Artemia*; a target for copper induced temporal delay of early development

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We have been using the brine shrimp *Artemia* to study nuclear processes during early development. The first twenty hours of development are characterized by a number of significant morphological and biochemical changes. The most dramatic nuclear events associated with these changes are the differential expression of nuclear proteins and the type and extent of glycosylation, e.g. histone H1, and steadily increasing levels of RNA transcription. The type and extent of nuclear glycosylation was monitored by Western blotting of nuclear extracts and measuring the binding of *Datura stramonium* (DSA), *Maackia amurensis* (MAA), *Galanthus nivalis* (GNA), and peanut (PNA) agglutins. Exposure of the embryos to 1 μM copper has no apparent affect on embryonic development. However, when exposed to 100 μM copper, there is a change in the morphology of the embryo and its development is temporally delayed for several hours. The delay does not result in any apparent long- term harmful affects to the embryo. There is a concomitant increased expression of a stress protein and diminished levels of hemoglobin, histone 1 and transcriptional activity in isolated intact nuclei. In addition, there are observable changes in the composition of the nuclear proteins and their glycosylation, e.g., diminished levels of H1 expression and changes in GNA binding activity. Thus, nuclear glycosylation presents itself as method for regulating gene transcription and as a biomonitor for the detection of non-lethal environmental stress. (Supported in part by GM 08239-09).

C5. Functional role of glycans

C5.1

Chitotriosidase: effect of chitin-containing glucans as macrophage stimulators

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Chitotriosidase (Ch), endo β-glucosaminidase, belonging to the protein family of chitinases, strikingly elevated in plasma of patients with Gaucher disease. Ch is most likely produced by the lipid loaden macrophages (Mph), called Gaucher cells. It may play a role in the degradation of chitin-containing pathogens. The aim: to study serum Ch activity during Mph stimulation by *chito*carboxymethylglucan (compare to carboxymethylglucan, both produced by Dr. J. Sandula) and in model of Mph overloading by non-digested lipids (Triton WR 1339). Serum Ch activity was determined fluorimetricaly (Guo *et al*, 1995). Ch activity was shown to elevate more than 1000-fold in plasma of Gaucher patients. In experiments in all models of Mph stimulation (zymosan, chito- or carboxymethylated glucan, Triton WR 1339) in CBA mice or Wistar rats 2-2.5 fold increase of serum Ch activity was shown. Similar results were obtained in the groups with different glucans studied (in the same dose of 25 mg/kg, i.p). Increased serum Ch was shown in the model of lipid-loaded Mph. One can *conclude* that serum Ch activity may serve as a valuable parameter for monitoring the efficacy of Mph stimulation in vivo.

Reference

Guo Y *et al* (1995) *J Inher Metab Dis* **18:**717-722

C5.2

The relationship between the degree of the rhamnan backbone substitution and the biological activity of *Pseudomonas syringae* lipopolysaccharides

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Lipopolysaccharides (LPSs) of three *Pseudomonas syringae* strains were characterized by the same lipid A and core oligosaccharide chemotype but different O-chain polysaccharide structures. The O-chain of strain CF-4 LPS is a linear D-rhamnan with a →3)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→2)-α-D-Rha*p*-(1→ trisaccharide O-repeat whereas in strains 218 and P-55 the same D-rhamnan is partially substituted with α-D-Fuc*f* (the degree of fucosylation is 35 and 85%, respectively). The biological activities of the LPSs, namely, mitogenic activity, including effect on T-cells proliferation, stimulation of cytokines release (NO, IL-10, IFN-γ, TNF-α) in mice and human cells etc., were comparatively studied. It was found that the level of the activities LPSs in human cells decreased or increased from 1.1 to 10.7 times in the following order: CF-4, 218, P-55. The opposite order observed in mouse cells. Since lipid A and core oligosaccharide have conserved structures, it was concluded that the degree of substitution of the D-rhamnan backbone with the D-Fuc side chain correlates with the level of biological activity of the LPSs.

The work was supported by INTAS-UKRAINE (grant 95-0142).

C5.3

Effects of the stage-specific expressing antigen Le^Y on implantation-related factors of mouse embryo

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Implantation is a complex process, and many factors involved may form a regulation chain of implantation. LeY oligosaccharide antigen is stage-specifically expressed on the embryo's surface during peri-implantation stage. Our previous work has shown that LeY antigen plays a vital role as a mediator between the uterus-embryo recognition in the initiation stage [1], and we also found that MMPs secreted from embryos are significantly inhibited by blocking LeY with specific antibody [2]. In this study, the effect of LeY on implantation-related factors, including MMPs, TIMP1, LIF, EGF were analyzed by MMP zymography, dot-blot immunoassay and RT- PCR methods. The results showed that all factors investigated execept TIMP1 were affected, after blocking LeY by specific antibody. The expression of MMP9 and MMP2 at the mRNA level and their secretion were obviously reduced; gene transcription of EGF was declined too; but the secretion and transcription of LIF were induced. The results indicate that LeY oligosaccharide on the surface of the embryo closely links with other implantation-related factors; it may be involved in the regulation chain of implantation as a signaling molecule.

References

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C5.4

N-Glycans stabilize protein conformation through their binding affinity for the hydrophobic protein surface

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N-Glycans have been found to directly promote protein folding [1] and stabilizing protein conformation [2]. To reveal the molecular basis for the stabilizing effect of N-glycosylation, the properties of N-glycan-modified human recombinant erythropoietin (EPO) species were compared. The stability and ANS binding of EPO species with different N-glycan structures revealed that the inner regions including the galactose residues of the highly branched N-glycans stabilize the protein conformation through their affinities for the hydrophobic protein surface accessible to ANS binding. When measured by means of surface plasmon resonance, the binding affinities of the immobilized N-glycans to *E. coli*-expressed EPO (nonglycosylated EPO) depended largely on their branching degrees; *i.e.*, tetraantennary N-glycans had a much higher affinity than bi- and tri-antennary ones. Thus it seems probable that highly branched N-glycans have extensive, hydrophobic regions.

References

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[2] Narhi LO *et al* (1991) *J Biol Chem* **266:**10624-10629

C5.5

Characterisation of the N- and O-glycans of apolipoprotein (a). Role of O-glycans in conferring protease resistance

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Apolipoprotein (a) is a multikringle domain glycoprotein that exists covalently linked to apo B100 of low density lipoprotein, to form the lipoprotein (a) particle, or as proteolytic fragments. Apo(a) and its fragments may promote atherosclerosis but the underling mechanisms are incompletely understood. The factors influencing apo(a) proteolysis are also uncertain. We have used exoglycosidase digestion and mass spectrometry to sequence the Asn(N)-linked and Ser/Thr(O)-linked oligosaccharides of human apo(a) and assessed the role of apo(a) O-glycans in protecting thermolysin-sensitive regions of the polypeptide. Apo(a) contained two N-glycans that accounted for 17% of the total oligosaccharide structures. The N-glycans were complex biantennary structures present in a mono- or disialylated state. The O-glycans were mostly (80%) NeuAcα2-3Galβ1-3GalNAc, with smaller amounts of disialylated and non-sialylated structures also detected. Removal of apo(a) O-glycans by sialidase and O-glycosidase treatment dramatically increased the sensitivity of the polypeptide to thermolysin digestion. These studies provide the first direct sequencing data for apo(a) glycans and furthermore indicate a novel function for apo(a) O-glycans that is potentially related to the atherogenicity of Lp(a).

C5.6

Metabolic changes in the double knock-out mice of GM2/GD2 synthase and GD3 synthase genes containing only GM3 ganglioside

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Using gene-knock-out mice, we have analyzed the regulatory functions of acidic glycosphingolipids. Mating of GM2/GD2 synthase gene knock-out mice and GD3 synthase gene knock-out mice resulted in the generation of new mutant mice that contain only GM3 (DKO). They are born almost normally and grown up with no marked macroscopic abnormalities. However, male DKO mice showed obesity in the adolescence (8-20 weeks), while female DKO mice showed marked obesity in the adulthood (32-40 weeks). Total cholesterol and triglycerade levels in DKO female mice were definitely higher than those in the wild type female. Blood pressure exhibited gradual elevation with aging in the male DKO mice, while that in the wild type did not significantly changed. The levels of blood sugar in the DKO male mice at food-taking state were significantly ($p<0.05$) higher than those in the wild type male. Surprisingly, they suffered from sudden death at the stage of young adult (12-18 weeks old). Taking these findings together, DKO mice have critical defects in the regulation of the lipid and sugar metabolism.

C5.7

Functional analysis of high-mannose type N-glycan of jack bean α-mannosidase

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Jack bean α-mannosidase, which probably is involved in the catabolism of glycoproteins in the vacuole, is itself a glycoprotein with one high-mannose type and one complex type N-glycan. We have already determined their detail structures and preliminarily reported an important function of the N-glycans involved in the subunit assembly. In this study, we show that the high-mannose type N-glycan linked to the 60 kDa subunit is clearly involved in the correct assembly of each subunit to recover the enzyme activity.
The denatured and deglycosylated α-mannosidase could not recover the enzyme activity, while the denatured and fully glycosylated α-mannosidase could recover the activity up to 80% of the native enzyme, after 8 hr-incubation in an appropriate renaturing buffer. Circular dichroism (CD) analysis showed that the deglycosylation of the enzyme by endo-H does not cause significant change in the secondary structure. On the contrary, intrinsic tryptophyl fluorescence measurement showed that the deglycosylation of the enzyme causes a significant change in the microenvironment of tryptophan residue(s). Furthermore, we have revealed that addition of high-mannose-type free N-glycan, which ubiquitously occurs in plant cells, could mediate the correct oligomerization to recover the α-mannosidase activity.

Reference

Kimura Y *et al* (1999) *Eur J Biochem* **264**:168-175

C5.8

Orp150/Grp170 is a sialyl-TF antigen expressing glycoprotein directly involved in NLS -dependent nuclear protein import

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Increased cell surface expression of the oncofetal Thomsen-Friedenreich antigen (TF, Galβ1-3GalNAcα-) is common in malignant and hyperplastic epithelia. Our previous studies have shown that a TF antigen-binding lectin from the common edible mushroom *Agaricus bisporus* (ABL) reversibly inhibits epithelial cell proliferation (*Cancer Res* 1993, **53:**4627) in association with inhibition of nuclear localization sequence (NLS)-dependent nuclear protein import (*J Biol Chem* 1999, **274:**4890). The purpose of this study is to investigate how ABL produces such an unique effect on nuclear protein import.
Using ABL-affinity purification, an ABL-binding 160~170 kDa protein was obtained from the HT29 cell cytosol extracts. This protein expresses sialyl-TF antigen as determined by specific sialidase/O-Glycanase treatment and lectin/Western blotting. N-terminal protein sequencing identified the protein as the oxygen (glucose) regulated protein150 (Orp150/Grp170), a highly diverged Hsp70-like protein. Introduction of an anti-Grp170 antibody to the digitonin semi-permeabilized nuclear transport system or depletion of Orp150/Grp170 inhibited the nuclear accumulation of a FITC-conjugated nuclear localization sequence peptide complex.
This is probably the first demonstration of an intracellular glycoprotein expressing sialyl-TF. The inhibition of nuclear accumulation of NLS-complex by anti-Orp150/Grp170 antibody suggests that this protein may be a novel nuclear transport factor.

C5.9

Novel aspects of protective action of carboxymethylated β-1,3-glucan and LPS

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Animals pretreated with yeast and bacterial polysaccharides show increased tolerance to several types of shock. Preliminary i.v. injection of carboxymethylated β-1,3-glucan (CMG) increased survival rate of rats with endotoxic shock, while acetylated low density lipoproteins (LDL) injected simultaneously with CMG decreased animals' survival to 60%. Earlier we showed that CMG competes with acetylated LDL for scavenger receptors in vivo and in vitro and preliminary CMG injections result in induction of scavenger function in nonparenchimal liver cells, which are known to play a major protective role against modified atherogenic LDL. This way CMG enhanced LPS clearance through scavenger receptors, decreased TNF-α production (4-fold) and reactive oxygen species generation. In this study we determined the content of endogenous oxysterols (oxidized derivatives of cholesterol) in rat peritoneal macrophages normal or tolerant to LPS. Incubation of control peritoneal macrophages with 25 μg/ml of LPS caused 4-fold higher TNF-α production response as compared to macrophages from tolerant animals. The concentration of 25-OH- and 27-OH-cholesterol in tolerant macrophages was significantly higher than in control (by 90 and 85%, respectively). Macrophage LPS tolerance is associated with intracellular accumulation of oxysterols and activation of scavenger receptors.

C5.10

A new rooting inhibitor glycan found during rice germination

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Rice, as a semi-aquatic plant, germinates with its own uniqueness: coleoptile is the only organ developed under submerged condition, with severely retarded growth of both leaves and root. This has been long ascribed to the evolution of the gaseous plant hormone, ethylene, from the seedlings when encountered flood stress. We have partially purified, from the culture medium, a substance (X, M_r ca 3,000) that exhibited the rooting inhibitory activity, in a dose-dependent fashion, with the effective concentration of less than 10^{-6} M. All the morphological changes observed in the presence of X were closely similar to those observed for the rice germ submerged in the stagnant water. These results are thus strongly indicating that X is involved in the biological consequence observed during germination of rice seeds in submergence. Major component of X was shown to be oligosaccharide(s) composed of arabinose, fucose, glucose, galactose, mannose, galactosamine and glucosamine, suggesting that X is presumably originated from glyco-protein(s). Most importantly, periodate oxidation was found to abolish completely the activity of substance X, whereas proteolysis did not. Relationship between the developmentally regulated expression of peptide:*N*-glycanase (PNGase) in the rice coleoptile and generation of substance X will be discussed.
(Supported by NSC-89-2311-B-001-069 (to TC)).

Reference

Chang T *et al* (2000) *J Biol Chem* **275:**129-134

C5.11

Lipopolysaccharides from *Sinorhizobium meliloti* CXM1-188 and its LPS-mutant Ts22

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Lipopolysaccharides (LPS) from *Sinorhizobium meliloti* CXM1-188 and its LPS-defective mutant Ts22 have been studied. Both strains induced nitrogen-fixing nodules but Ts22 lessened nodulation competitiveness in comparison with parent strain. LPS were isolated by phenol-water procedure, purified cetavlon and ultracentrifugation (105,000 g). It has been shown by SDS-PAGE that CXM1-188 synthesizes LPS1, the O-antigen-containing form of LPS, and LPS2 which corresponds R-LPS. Mutant Ts22 showed the ability to synthesize only LPS2. LPS from two strains contained glucose (the predominant sugar), galactose, mannose and non-identified deoxy-hexose, uronic acids, glucosamine, 2-keto-3-deoxyoctonate (KDO) and such fatty acids (fa): 3-OH C14:0 (main component), 3-OH C15:0, 3-OH C16:0, 3-OH C18:0, C18:1, C18:0 and non-identified hydroxy fa X. Ts22 LPS differed from CXM1-188 LPS by the higher relative content of KDO, galactose and C18:0. Thus, a single Tn5-insertion was shown to induce both the defect of LPS synthesis and the decrease of nodulation competitiveness. That points the importance of the presence of intact LPS for manifestation of nodulation competitiveness of *S. meliloti*.

C5.12

Analysis of suppression mechanism of intrinsic metastasis potential in human lung carcinoma cells by overexpression of the *N*-acetylglucosaminyltransferase-IVa (GnT-IVa) gene

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We have reported a possible suppression effect of intrinsic metastasis potential in human lung carcinoma cells by overexpression of the GnT-IVa gene [1]. The GnT-IVa transfectant indicated highly suppressed metastasis potential, lower invasiveness into a matrigel and slower growth rate than parental cells, although carbohydrate associated adhesion molecules of cell surface showed no significant difference between GnT-IVa transfectant and control cells. The present study was to further understand the suppression mechanisms of metastasis by overexpression of GnT-IVa gene. We compared the tumorigenicity of GnT-IVa transfectants and parental cells after s.c. inoculation into the flank of nude mice. Results indicated that growth of parental cells were progressive manner, as expected. On the other hand, GnT-IVa transfectants could not form a nodule. As possible factors of effective on metastasis, differences in cell attachment and proteolytic activity to extracellular matrices of two cell systems, and the difference of immunoreaction contributing metastatic nodule formation ability will be presented.

Reference

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C6. Glycobiology in evolution

C6.1

The evolution of cytidine monophosphate-*N*-acetylneuraminate hydroxylase

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The structural diversity of sialic acids arises by the enzymatic derivatisation of *N*-acetylneuraminic acid (Neu5Ac). The predominant occurrence of sialylated glycoconjugates in deuterostome animals raises questions regarding the evolution of the enzymes of sialic acid biosynthesis, in particular those involved in Neu5Ac-derivatisation. The formation of *N*-glycolylneuraminic acid (Neu5Gc), a common sialic acid variant, occurs by the hydroxylation of CMP-Neu5Ac. CMP-Neu5Ac hydroxylase is a cytochrome b_5-dependent oxygenase which contains a Rieske [2Fe2S] centre and an exchangeable iron cofactor. A comparison of the properties and sequence of CMP-Neu5Ac hydroxylase from various animals shows that with the exception of the loss of a membrane-binding domain, the enzyme has been largely conserved in the course of evolution from echinoderms to mammals [1]. Interestingly, sequence alignments suggest that the early evolution of CMP-Neu5Ac hydroxylase occurred along a different lineage to all known prokaryotic and eukaryotic Rieske oxygenases [2].

References

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[2] Schmidt CL and Shaw L (2001) *J Bioenerg Biomemb* **33**:9-26

C6.2

Evolution of glycosyltransferase genes in eukaryotes

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The past few years have seen rapid advances in sequencing the genomic DNA of human, *Caenorhabditis elegans*, and so on. As a result, a large number of novel glycosyltransferase genes have been discovered from those genome sequences. How did they increase their family members during the genome evolution? To presume the evolutional pathway of glycosyltransferases, we have analyzed using the molecular evolutionary analysis. We conducted molecular evolutionary analyses on 55 glycosyltransferase genes. The phylogenetic analysis revealed that gene duplications within glycosyltransferase gene families occurred in early vertebrate evolution, and gene duplications among glycosyltransferase gene family occurred prior to appearance of Deuterostomes. These analyses suggested that the glycosyltransferase gene increased their numbers through gene duplications and genome duplications. Comparison of evolutionary rates indicated that the glycosyltransferase tend to evolve slowly than other genes, and the evolutionary rates changed within each of the glycosyltransferase gene families. These results indicate that the increase of glycosyltransferase gene allows them amino acid change and permit to create the variety of enzyme activity.

C6.3

Teosinte *(Zea diploperennis)* and corn (*Zea mays*) coleoptile lectins: specificity for β-galactose preserved through evolution

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Current evidence suggests that teosinte through homeotic sexual translocations gives rise to corn [1]. In order to identify evidences of their common origin we purified and characterized the lectin from teosinte (TCL) and corn (CCL) coleoptiles. TCL and CCL are 92 and 88.7 kDa proteins, respectively. They showed a similar amino acid composition by Glu, Asp, Gly, and Ser; and in minor proportion Met and Cys. Only TCL is formed by two molecular isoforms. The N-terminal of both lectins is blocked, but the amino acid sequence of the lectin, determined from tryptic peptides by MALDI-TOF, indicated they showed homology with a 49 kDa protein from *Arabidopsis thaliana* and a NADPH-dependent reductase from *Zea mays*. These lectins showed hemagglutinating activity toward several animal erythrocytes, and they interact specifically with the OH on C4 from galactose residues; β-Gal residues are better recognized than α-Gal forms. TCL possesses an extended sugar specificity since higher concentrations of lactose, than CCL, were necessary to inhibit the lectin. Our results indicated that both lectins possess preserved sugar binding properties through evolution, suggesting that in effect, corn is the result of teosinte evolution. (Financed in part by CONACyT and DGAPA-UNAM, México).

Reference

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C6.4

More structures than cells? The N-glycans of *Caenorhabditis elegans*

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For a preliminary characterisation of the Asn-linked glycans of *C. elegans*, worms were at first boiled to inactivate glycosidases and then glycopeptides were purified by serial chromatography. Glycans were released by PNGase A and analysed by MALDI-TOF MS and RP-HPLC of PA-sugars. The (neutral) N-glycans fell into four groups: (1) oligomannosidic N-glycans; (2) core-α1,6-fucosylated N-glycans such as Manα3(Manα6)Manβ4GlcNAcβ4(Fucα6)GlcNAc; (3) minute amounts of a complex type N-glycan with one terminal GlcNAc-residue; and (4) a range of fucosylated paucimannosidic N-glycans containing three to five mannose residues, up to four fucose residues and up to two *O*-methyl groups. Monosaccharide analysis of the N-glycan pool indeed indicated the presence of 2-*O*-methyl fucose and 3- or 4-*O*-methyl mannose besides fucose and mannose. N-glycans with two or three fucose residues essentially proved resistant to α-mannosidase regardless of the degree of methylation indicative of mannose-residues being substituted by fucose. Thus, in accordance with data obtained by MS-MS [1], some fucose residues appear to be linked to mannoses. The structural variety is finally increased by *O*-methylation.

Reference

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C6.5

Expression of glycoproteins containing Galα1-4Gal in pigeon

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Galα1-4Gal on glycoproteins is uncommon in Nature. Mammals express Galα1-4Gal mostly on glycolipids (e.g., Gb3, P_1 antigen, etc.), but rarely on glycoproteins. However, we have discovered that the major glycoproteins from pigeon egg white were rich sources of Galα1-4Gal, and identified their N-glycan structures. The presence of Galα1-4Gal is limited even among birds, because Galliformes (e.g., chicken) and Anseriformes (e.g., duck) have no Galα1-4Gal antigenic activity. We examined expression patterns of glycoproteins with Galα1-4Gal in pigeon to gain insight of the presence of Galα1-4Gal. The western blot with GS-I lectin (terminal α-Gal specific) and anti-P_1 mAb revealed that pigeon liver, heart, lymphocytes, and serum as well as egg yolk contained Galα1-4Gal on various glycoproteins, suggesting that the (Galα1-4Gal)-containing glycoproteins are widely distributed in the pigeon body. One of the major glycoproteins from pigeon plasma, which has strong P_1 antigenic activity, was isolated by gel filtration and revealed similar molecular properties of heavy chains of chicken IgG (alias IgY). The tissue independent expression of Galα1-4Gal on glycoproteins in pigeon and the total absence of Galα1-4Gal in some birds suggest that the evolutional pressure to express Galα1-4Gal have occurred during the diversification of modern birds.

References

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C7. Glycobiology of animals

C7.1

Distinct metabolic pathways of glycolipids in the cells immortalized from murine thymus

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Mammalian tissues usually contain the tissue-characteristic glycolipids, some of which are implicated in their functions, but cells composing of tissues are not homogenous depending on their roles in the respective tissues. By means of the SV-40-mediated immortalization, we established three cell lines, IWMTH-E, -I and –F, which were morphologically distinct from each other, from murine thymus, and the glycolipid composition and the related enzymes were determined by TLC-immunostaining and RT-PCR. In accord with the major glycolipid pathways in murine thymus, glycolipids belonging to the globo- and ganglio-series were expressed in all of the cells, but the modification of the carbohydrate chains of glycolipids was different among the cells. The metabolic pathways of the globo-series were terminated at Gb_3Cer in IWMTH-E, Gb_4Cer in IWMTH-I, and Forssman antigen in IWMTH-E, respectively. Gg_3Cer and GD1a were detected in IWMTH-I and –F, but not in IWMTH-E, which contained glycolipids with rather shorter carbohydrate chains than those in the other cells. The final products of the ganglio-series glycolipids in IWMTH-E were GM2 and II^3SO_3-Gg_3Cer, and sulfoconjugation of glycolipids was only observed in IWMTH-E. The difference in the glycolipid compositions was due to the anabolic enzymes, and the cells having the distinct carbohydrate chains should provide a clue on the functional significance of glycolipids in murine thymus.

C7.2

The freshwater prawn *Macrobrachium rosenbergii* lectin share homology with the immunoglobulin superfamily fold

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In the hemolymph of freshwater prawn *Macrobrachium rosenbergii*, there is a 19 kDa glycosylated lectin (MrL) specific for *N*-acetylated sugar residues and particularly *O*-acetyl-sialic acid. Previous works demonstrated the participation of this lectin in the immune recognition. With the aim at evaluate its participation in defense mechanisms, in this work we determined the chemical and structural properties from MrL were evaluated. MrL is a dimeric glycoprotein with 11% of glycans by weight; chemical characterization of purified glycopeptides indicated that the lectin is composed of 2 N-linked glycans: $Man_3GlcNAc_2Gal_{1.3}$ and $Man_3GlcNAc_{2.8}$, and probably 1 O-linked glycan: $Gal_3GlcNAc_3$-$GalNAc_{0.4}NeuAc_{0.2}$. The amino acid composition shows 65% of acid amino acids; the sequence, as deduced by MALDI-TOF mass spectrometry, shows homology with the hyperglucemic hormone from *M. rosenbergii* (51%) and the variable portion of the κ (22%) and λ (27%) chains from human antibodies. The combination of circular dichroism and secondary structure prediction show a content and organization very similar to the immunoglobulin superfamily fold. This homology have been observed in lectins from *Limulus polyphemus*, and opens new questions about the evolution of the lectin. (Financed by CONACYT, PAPIIT and PAEP-UNAM, Mexico).

C7.3

N-Glycosylation of chicken prolactin with no typical consensus amino acid sequence N-X-S/T

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Prolactin (PRL) is a pituitary hormone, which controls a variety of behaviors of vertebrates. The glycosylated isoforms of prolactin (GPRL), which are widely distributed in pituitary and serum may have an effect on half-life, conformation and the biological activity of the hormone. The chicken PRL cDNA of Ross strain was isolated and sequenced. The predicted amino acid sequence varied from the one in GenBank, but did not contain the typical N-X-S/T site for N-glycosylation. However, the chicken PRL from pituitary and serum, recognized by the specific anti-chicken PRL antibody, expressed N-glycosylated isoform. The Neuro2a cells transfected by chicken PRL gene also expressed two monomeric isoforms of PRL. The GPRL, purified from chicken pituitary, is recognized by concanavalin A and *Ricinus communis* lectins. However, it did not react with sialic acid specific lectins. The GPRL isoform was several times less effective in stimulating the proliferation of rat Nb2 lymhoma cells than the nonglycosylated PRL. The results described above support Corcoran's and Proudman's hypothesis [1] of an alternative site for N-glycosylation of proteins in avian. (Financial support was provided by the R. Koch Fundation, Warsaw, Poland).

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C7.4

Variation of glycosylation during the estrous cycle of the hamster

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Oviductins are a family of glycoproteins synthesized and secreted by non-ciliated oviductal cells. Immunocytochemical studies using antibodies recognizing a glycosidic epitope, or the protein core, suggested that glycosylation of hamster oviductin is under hormonal control. The labelling pattern of the glycosidic epitope varied throughout the estrous cycle with the strongest labelling detected at the stage of estrus. However, the pattern of the polypeptide epitope did not vary significantly throughout the five stages of the estrous cycle. In addition, mRNA expression of the hamster oviductin gene remained constant throughout the estrous cycle. In the present study, the activities of several glycosyltransferases assembling mucin-type O-glycan chains, were investigated throughout the five stages of the estrous cycle. In particular, core 2 β6-GlcNAc-transferase activity showed a distinct pattern throughout the estrous cycle with high activities at the stages of proestrus and estrus and low activity at the stage of diestrus 1. This corresponded to the glycosylation of hamster oviductin. An increase in glycosyltransferase activity at the time of ovulation suggests that glycosylation of oviductin, may be a requirement for fertilization. (Supported by the Canadian Institutes of Health Research and the Canadian Cystic Fibrosis Foundation).

C7.5

Towards understanding the phosphorylation of β1,4-galactosyltransferase

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Regulation of glycosylation processes is poorly understood. One of the main control mechanisms in the cell is phosphorylation/dephosphorylation. In spite of the fact that many secreted proteins are phosphorylated, neither the kinases involved nor their subcellular localisation are known. Indications that β1,4-galactosyltransferase is phosphorylated already exist [1] and others identified a co-purifying possibly relevant cdc2-like kinase, named galactosyltransferase-associated p58 protein kinase (subsequently re-named PITSLRE β1) [2]. Although PITSLRE kinases were later shown to be localised to the nucleus and cytoplasm with apparent roles in regulation of the cell cycle and apoptosis, recent data show that PITSLRE β1 is present in a Golgi membrane fraction [3]. We are now re-examining the phosphorylation of β1,4-galactosyltransferase. To this end, both bovine mammary gland β1,4-galactosyltransferase and PITSLRE β1 were cloned and expressed in *E. coli*; respective antibodies are being used in the study of their interactions.

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C7.6

Molecular basis of the neural anti-HRP epitope in *Drosophila melanogaster*: Identification of core α1,3-fucosylated glycans and cloning of the requisite fucosyltransferase cDNA

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For many years polyclonal antibodies raised against the plant glycoprotein horseradish peroxidase have been used to specifically stain the neural and male reproductive tissue of *Drosophila melanogaster*. This epitope is considered to be of carbohydrate origin, but no glycan structure from *Drosophila* has yet been isolated that could account for this cross-reactivity. Here we describe the identification by MALDI and HPLC of two N-glycans from adult flies that, as already detected in other insects, carry both α1,3- and α1,6-fucose residues on the proximal core GlcNAc. Moreover, we have isolated a cDNA encoding α1,3-fucosyltransferase homologues from *Drosophila*, which was found to direct expression of core α1,3-fucosyltransferase activity when engineered in *Pichia pastoris*. The recombinant enzyme preferred as substrate a dansyl glycopeptide carrying a biantennary core α1,6-fucosylated N-glycan carrying two non-reducing *N*-acetylglucosamine residues ($GnGnF^6$) over the same structure lacking a core fucose residue (GnGn). This enzyme was also shown to be able to modify defucosylated N-glycan structures, correlating with the acquisition of binding to anti-horseradish peroxidase. Moreover, anti-HRP whole-mount staining of *Drosophila* embryos could be inhibited upon addition of bromelain glycopeptides carrying $M0XF^3$ structures strongly suggesting that core α1,3-fucose may be the neural *Drosophila* epitope.

C7.7

UDP-GlcNAc and UDP-GalNAc pyrophosphorylases: Relation of the two proteins and their role in glycosylation and its regulation

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GlcNAc and GalNAc are major sugars in the structures of N- and O-linked oligosaccharides, proteoglycans and glycolipids. Thus the enzymes involved in the activation of these sugars are of prime interest and importance. In addition, the availability of these enzymes would provide valuable tools for the preparation of photoactive and radioactive sugar nucleotides. We purified the UDP-GlcNAc pyrophosphorylase from pig kidney and found that the enzyme had equal activity for the synthesis of UDP-GalNAc (from UTP and GalNAc-1-P) as for the synthesis of UDP-GlcNAc (from UTP and GlcNAc-1-P). The purified enzyme had 2 protein bands of 64 and 57 kDa. These two proteins had the identical amino acid sequence except for a 17 amino acid insert near the carboxy terminus in the 64 kDa protein. Each protein was cloned and expressed in an *E. coli* expresssion system. The 64 kDa band synthesized UDP-GlcNAc about 8 times faster than UDP-GalNAc, whereas the 57 kDa protein synthesized UDP-GalNAc about 4 times faster than UDP-GlcNAc. Azido-UDP[^{32}P]-GalNAc was used to label the substrate binding site of the 57 kDa protein. Protease digestion of the labeled protein gave a labeled 24 amino acid peptide located between residues 216 and 240. Four amino acids in this peptide were highly conserved and mutation of Gly^{222} or Gly^{224} to Ala resulted in complete loss of enzymatic activity. The distribution of the 57 and 64 kDa proteins in various rat tissues is currently being examined as well as the effect of glucosamine feeding on these activities. (Supported by NIH HL17783).

C7.8

Both β-1,4-galactosyltransferase and β-galactosidase activities are enhanced in AZT-treated K562 cells

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3'-Azido-3'-deoxythymidine (AZT, zidovudine) is one of the primary drug used in the treatment of immunodeficiency virus (HIV) infection. Among the various reported biochemical effects, it has also been shown that AZT suppresses the incorporation of both sialic acid and galactose into proteins [1]. In view of these relevant observations, in the present study we focussed our attention mainly on the determination of both β-1,4-galactosyltransferase and β-galactosidase activities either in AZT-treated human erythroleukemia K562 cells and in control cells. Then, K562 cells were grown in the absence or in the presence of 40 μM AZT for 3 h at 37 °C and after cell lysis appropriate aliquots were subjected to an ELISA-based assay [2]. Our results showed that both β-1,4-galactosyltransferase and β-galactosidase activities were enhanced in AZT-treated K562 cells, being the increase about 30% in the former case and 12% in the latter case, respectively. Although previous data [1] suggest that AZT does not alter protein biosynthesis or protein secretion, but inhibits protein glycosylation mainly by blocking the nucleotide sugar import into the Golgi complex, our findings suggest that some glycosyltransferase or glycosidase activity might differently be modulated in the presence of AZT. This evidence could partly explain the alteration induced by AZT on glycosylation patterns.

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C8. Glycobiology of humans

C8.1

Glycoconjugate abnormalities in patients with congenital dyserythropoietic anemia (CDA) type I-III

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Glycoconjugate abnormalities of erythrocyte membrane glycoconjugates consisting of hypoglycosylation of band 3 and glycophorin A as well as accumulation of glycolipids including Lc3Cer, nLc4Cer and polyglycosylceramides have been found only in patients with CDA-II (HEMPAS). Recently, however, we have described a CDA-I patient in Poland with identical, though less pronounced, glyco-conjugate abnormalities [1]. We now report on similar findings in three more patients with CDA-I. In addition, we have examined erythrocytes from three patients with CDA-III and also found glycoconjugate abnormalities affecting, however, almost exclusively glycolipids.

Reference

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C8.2

Glycosidase treatment on serum lipoproteins: Mobility on the electrophoresis and immunoreactivity to apolipo protein-antibodies

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It is believed that one of the factors of atheroma formation in arterial wall is the presence of unusual LDLs, especially, oxidized LDL which is a product of oxidation of the lipid portion of LDL. The protein portion of LDL is apolipoprotein B100 ($ApoB_{100}$) which molecular weight is reported as 51.3 x 10^4 dalton and the amino acid number is 4536. And also the existence of 16 oligosaccharide chains is reported. One half of the number of oligosaccharides is high mannose type and the other half is mono- or di-sialo type. We have a working hypothesis that the normal LDL is incorporated via the LDL receptor into the cells but the unusual LDL which has semihydrolysed oligosaccharide chains on $ApoB_{100}$ is not recognized by the LDL receptor and not incorporated. These unusual LDLs are accumulated in intercellular space of the arterial wall and attacked by macrophages. Method: Gel-electrophoresis and lipid stainning. Immunoelectrophoresis. Results: (1) Sialidase treatment on the LDL results in the decreasing mobibity on electrophoresis and normal reactivity with ApoB100-antibody; (2) Mannosidase treatment on the LDL results in the decreasing mobility and weak reaction with ApoB100-antibody; (3) *N*-Acetylglucosaminidase treatment on the LDL results in essentially the same type of changes as found for mannosidase treatment; (4) Fucosidase treatment results in no effect on the LDL.

C8.3

Glycosylation abnormalities in red cell membranes of heterozygous parents of a patient with congenital dyserythropoietic anemia (CDA) type II

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CDA type II is an autosomal recessive disease. Recently we have found a family with CDA type II in Poland and used this opportunity to examine red cell glycoconjugates from the propositus and of his healthy but presumably heterozygous parents. Glycosylation abnormalities were clearly seen in red cell membranes of the parents and involved hypoglycosylation of band 3 and accumulation of Lc3Cer and of polyglycosylceramides. Complexity of the latter was reduced. The abnormalities in band 3 - and ceramide bound polylactosaminoglycans showed dosage effects and in quantitative terms amounted to about 50% of those found in red cell membranes of the propositus. On the other hand, though both Lc3Cer and nLc4Cer were elevated 142-fold and 551-fold respectively in red cell membranes of the propositus only the former GSL was elevated in red cell membranes of the parents. This is the first report on glycoconjugate abnormalities in healthy CDA II heterozygotes.

C8.4

Glycolipids of human leukemic cells

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Leukemia arises following malignant transformation and subsequent clonal expansion of a single hematopoietic progenitor that is unable to mature. We report on glycoconjugates in M0, M1, M2, M3 and M5 leukemic cells from 24 patients with acute myeloid leukemia. These cells represented successive differentiation stages from the most primitive M0 cells to mature neutrophils and monocytes. Transition from M1 to M3 cells and normal granulocytes was associated with the accumulation of LacCer, Lc3Cer, nLc4Cer and disappearance of GM3 that was replaced by NeuAcnLc4Cer. These changes were associated with the formation of granules that were rich in GSLs. Monocytic differentiation was associated with the appearance of GM3, Gb3Cer and Gb4Cer. Immature neutrophils (metamyelocytes), very rich in glycolipids, were present in blood from all four patients with chronic myeloid leukemia. Free ceramides and ceramide portions of GSLs had exactly the same composition in all immature and mature cells of neutrophilic lineage with d18:1 sphingoid base and C16:0 fatty acid as major components, and C24:0 and C24:1 fatty acids as minor components. Contents of total, cellular protein-linked carbohydrates dramatically increased from 3.8 (±1.2) nmol/10^6 cells in M1 cells to 18.3 (±1.5) nmol/10^6 in mature neutrophils. Total carbohydrates of glycosphingolipids followed the same pattern.

C8.5

cDNA cloning and characterization of a novel galactose 3-*O*-sulfotransferase specific for O-linked core 1 structure

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Sulfation is one of the most extensive modification in O-linked and N-linked glycans and plays important roles in various biological events. Based on the amino acid sequence of human cerebroside 3′-sulfotransferase (CST), we isolated a cDNA clone from human testis cDNA library. The cDNA contained an open reading frame for a protein of 486 amino acids with a type II transmembrane topology. The amino acid sequence displayed 33 % and 39 % identity to human CST and human glycoprotein-specific Gal:→3′ sulfotransferase (GP3ST), respectively. The coding region was encoded in three exons and localized in chromosome 7q22. Crude membrane fraction derived from COS-7 cells transfected with a vector containing the cDNA exhibited a Gal:→3 sulfotransferase activity, named GP3ST-2. Galβ1→3GalNAcα1-*O*-pNP and Galβ1→3(GlcNAcβ1→6)GalNAcα1-*O*-pNP were good substrates for GP3ST-2, whereas Galβ1→3/4GlcNAc were poor ones. Using asialofetuin as substrate, sulfation exclusively occurred in O-linked glycans. These results suggest that GP3ST-2 is highly specific for O-linked Galβ1→3GalNAcα1 structure, and that the substrate specificity is quite different from those of CST and GP3ST, which recognize galactosylceramide and Galβ1→3/4GlcNAc as good substrates, respectively. Northern blot analysis revealed that GP3ST-2 gene is expressed in human various tissues. These results suggest that GP3ST-2 is the third member of a Gal:→3 sulfotransferase family, and responsible for sulfation of O-linked glycans.

C8.6

Human plasma contains endogenous 64-kDa trans-sialidase

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Incubation of low density lipoprotein (LDL) with autologous blood-plasma-derived serum leads to loss of sialic acid by lipoprotein particles. Desialylated LDL became capable to accumulate cholesteryl esters in human intimal smooth muscle cells and macrophages. The gel-chromatography demonstrated the presence of desialylating activity in both LDL and lipoprotein-free serum. Desialylation-causing enzyme was isolated by affinity chromato-graphy on a sialooligosaccharide-Sepharose. Sorbent-bound protein fraction was eluted with sialic acid, electrophoresis of eluted material in denaturating conditions reveals only one protein band 64 kDa. Isolated enzyme can remove sialic acid from purified LDL, fetuin, gangliosides, and PAA-glycoconjugates in presence of sialic acid acceptor; sialidase activity (in absence of an acceptor) was negligible. Substrate preference for the enzyme reduced in range 2-6 > 2-3 >> 2-8, fucosylated type 1 and type 2 structures are also good acceptors. pH optima revealed for the enzyme were 5.0 and 7.0. Calcium and magnesium ions stimulated the trans-sialidase activity at millimolar concentrations. Thus, trans-sialidase was isolated from human blood serum and partially characterized.

C8.7

Glycosylation of α_1-acid glycoprotein in human seminal plasma

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Human α_1-acid glycoprotein (AGP), also known as orosomucoid, is a normal constituent of blood, saliva, amniotic fluid and seminal plasma. The concentration of AGP in plasma is increased several fold during inflammation. It is a highly negatively charged glycoprotein containing five N-linked glycans that differ in branching, extent of fucosylation and sialylation in dependence of the pathophysiological condition.
Preliminary studies using crossed affinoimmunoelectrophoresis (CAIE) with AAL and ConA indicated that the glycosylation of AGP in seminal plasma differed from those in blood plasma. In this study we have applied CAIE with various lectins to investigate the glycosylation of seminal plasma AGP in men living in infertile couples. Large individual differencies were found in total AGP concentration and in degree of fucosylation and sialylation of the glycans. Fucosylated AGP glycoforms were only detected at AGP concentrations below <0.12 mg/ml. The absence of fucosylation at high concentrations coincided with an increased α(2-6)/α(2-3) sialic acid ratio as determined by CAIE with MAA and SNA. These results suggests that large individual changes exist is the relative activities of α3-fucosyltransferase, α(2-3)- and α(2-6)-sialyltransferases in the human prostate, the source of seminal plasma AGP.

C8.8

The IGF cascade in children with CDG 1A

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Patients with congenital disorders of glycosylation (CDG) exhibit growth failure. Insulin-like Growth Factors (IGFs) play an important role in somatic growth. In the serum, IGFs are found primarily bound to IGF binding protein-3 (IGFBP-3) and an acid-labile subunit (ALS) in a ternary complex which stabilizes the IGFs and regulates their delivery to target tissues. Glycosylation has been shown *in vitro* to affect the formation of functional ternary complexes and the stability of its components. In this first *in vivo* study, the components of the IGF cascade were analyzed in serum from children with CDG 1A (phosphomannomutase deficiency) and compared to a group of age-matched controls. Analysis of ALS by ELISA show reduced levels of free and total ALS in children with CDG 1A. Immunoblot analysis of ALS shows multiple bands suggestive of underglycosylation in children with CDG 1A. Serum levels of IGF-1 and IGF-2 were lower in CDG 1A. However, free IGF-1 was not significantly different. IGFBP-3 levels measured by ELISA, IRMA, immunoblotting and western ligand blotting were significantly lower in CDG 1A but not due to proteolysis. In conclusion, multiple components of the IGF ternary complex are reduced in the serum of children with CDG 1A warranting further functional studies.

C8.9

Concanavalin A in the diagnosis of trachoma

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Trachoma is an ocular chronic infection with serious sequelae due to repeated infection with *Chlamydia trachomatis*. It is the causative agent of severe conjunctivitis and the alteration of corneal epithelium and blindness. *C. trachomatis* contained a major glycoprotein in the external membrane of the bacteria, with high mannose N-glycans. In this work was used the Glc/Man specific lectin Con A-FITC labeled and revealed under Wood light, as a tool in the diagnosis of trachoma. Our results indicated that 1 µg/ml of Con A-FITC interact specifically with the tarsal surface of trachoma, where is located the bacteria. Until now the 19 cases of active trachoma studied were Con A positive, the presence of the bacteria was confirmed by isolation and PCR. 66 individuals showing different corneal pathologies such as conjunctivitis were ConA-FITC negatives. Our results indicated that the presence of high mannose glycans of the bacteria is not modified during infection, suggesting a possible role in evasion of response from the host; moreover, the use of Con A is proposed as alternative tool for trachoma diagnosis of trachoma. (This work was partially financed by SIBEJ-CONACYT. México).

C8.10

Investigation of the serological reactivity of the antibodies produced against *Citrobacter braakii* O37 with human and horse erythrocytes

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Molecular mimicry was found for *C. braakii* O37 which shares epitopes with both horse and human erythrocytes. Rabbit serum against *C. braakii* O37 bacterial cells reacted in agglutination test with horse and human erythrocytes but not with sheep, bovine, pig, cat, dog, and chicken erythrocytes. This project concerns the molecular basis of this cross reactivity. Experiments involved analysis of epitopes of both horse and human erythrocyte membranes recognized by the affinity purified antibodies anti-*C. braakii* O37 lipopolysaccharide (LPS). Results showed that band 3 glycoprotein of human erythrocyte is recognized by the specific antibodies purified on *C. braakii* O37 LPS affinity column. These antibodies immobilized on affinity column allowed to isolate 98 kDa protein from the triton-solubilised human erythrocyte membranes. Parallelly, the structural studies of the carbohydrate domain of *C. braakii* O37 LPS were performed. The O-specific polysaccharide is built up of trisaccharide repeating unit, composed of sialic acid, glucosamine and fucosamine residues. The core oligosaccharide is a novel type of LPS core but structural details remain to be determined. The initial immunochemical results indicate that both carbohydrate part of LPS and erythrocyte band 3 glycoprotein share the common epitope.

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C9. Glycobiology of parasites

C9.1

Glycosylation mutants in *Trypanosoma cruzi*

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Mutagenized cultures (*N*-methyl-3-nitro-1-nitrosoguanidine/ethane methane sulphonate) of *T. cruzi* epimastigotes were treated with the toxic lectins Concanavalin A (ConA) and Ricin (Ric) and the mutants isolated by selecting cells resistant to killing or agglutination by the lectins. Two mutants resistant to ConA (ConA1 and ConA2) and one resistant to Ric (Ric1) were characterized. There was no morphological difference among the mutant cells compared to the wild type. All the mutant cells showed reduced lectin binding as shown by lectin fluorescence microscopy and flow citometry. However, the ConA resistant cells exhibited different phenotypes as indicated by the differential binding of Wheat Germ Agglutinin (WGA) and Ricin. Blots of SDS-PAGE resolved *T. cruzi* epimastigotes proteins probed with ConA or Ricin showed clearly that many proteins lost their ability to bind the lectins and resembled gels of deglycosylated wild type cells. Taken together these results suggest a defect in the glycosylation pathway in the mutant cells. Confirmation on this point was obtained comparing glycosyltransferase activities of wild type and mutant cells. The level of β-1,4-Gal-transferase in the Ric mutant was reduced more than 20-fold compared to the wild type. Regarding the ConA resistant cells, we found in the ConA1 a reduced level of GlcNAc-transferase activity (10 fold), but no change in the ConA2 mutant, thus indicating the deffect is in a different step of the glycosylation pathway. Structural analysis of cell surface glycans in both mutant cell lines compared to the wild type should provide evidence of the altered step.

C9.2

Characterization of small, antigenic glycosphingolipids from *Schistosoma mansoni* eggs

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Pyridylaminated carbohydrate moieties of glycosphingolipids from eggs of the human parasite, *Schistosoma mansoni*, were analyzed by methylation analysis, partial hydrolysis, MALDI-TOF-MS and nano-ESI-MS. Apart from the large, highly fucosylated structures previously described [1] we found short, oligofucosylated species containing three to four HexNAc residues. These glycolipids exhibited Fuc(1-3)GalNAc(1-4)GlcNAc-, Fuc(1-3)GalNAc(1-4)[Fuc(1-3)]GlcNAc-, and GalNAc(1-4)[Fuc(1-3)]GlcNAc-units as terminal structures. In addition, a Lewis X-containing pentasaccharide was identified, which also occurs in cercarial glycolipids [2]. The different species were characterized as to their reactivity with mAb M2D3H. Fuc(1-3)GalNAc(1-4)GlcNAc- and Fuc(1-3)GalNAc(1-4)[Fuc(1-3)]GlcNAc-epitopes were recognized by M2D3H, whereas terminal GalNAc(1-4)[Fuc(1-3)]GlcNAc- and Lewis X-units did not react. The terminal structures were further tested for their contribution to the pronounced antigenicity of egg glycolipids employing binding assays with *S. mansoni* infection sera.

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C9.3

Structure-activity studies on glycosylphosphatidylinositol anchors of *Plasmodium falciparum*

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The glycosylphosphatidylinositol (GPI) anchors of *P. falciparum* can induce proinflammatory cytokine responses in macrophages and this has been implicated in malaria pathogenesis. People in endemic areas elicit anti-GPI antibody response in an age-dependent manner. While the majority of children under 3-4 years either lack or contain low levels of short lived antibodies, most children above 7-8 years and all adults contain relatively high levels of persisting antibodies, which strongly correlated with resistance to severe malaria. To determine the active portions of GPIs, structurally defined components were prepared by chemical and enzymatic degradations. The purified compounds were studied for the induction of TNF-α secretion by *in vitro* cultured macrophages, and for the anti-GPI antibody reactivity. The data demonstrate that the distal fourth mannose residue and *sn*-1 and/or inositol acyl substituents are crucial for the induction of TNF-α. Interestingly, the distal fourth mannose is also required for the efficient binding of anti-GPI antibodies; the removal of the mannose residue caused ~50% reduction in the activity. However, the glycan core containing all four mannose residues was unable to inhibit the antibody reactivity. The removal of *sn*-2 acyl group also resulted in a significant loss of the antibody reactivity, suggesting that the antibodies are directed against both the terminal mannose residue and the PI moiety.

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C9.4

Structural analysis of glycans from the trematode parasite schistosome and its intermediate host

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The trematode, schistosome, is a digenetic parasite which propagates through alternating mammalian and molluscan hosts. Previous work on *Schistosoma mansoni* have demonstrated a significant stage specific modulation of its glycosylation profiles, including terminal multi-fucosylation and xylosylated N-glycan cores. Interestingly, cross reactivities have been demonstrated among glycotopes from different species and various tissue or fluids from the snail host, *Biomphalaria glabrata*. We present here structural evidence that the N-glycans from the major glycoproteins of the hemolymph from *B. glabrata* likewise carry core xylosylation and α3,α6-fucosylation, giving interesting comparison to those of *S. mansoni* in several ways. Strikingly, most of the N-glycans, from truncated pauci mannose to high mannose types are naturally *O*-methylated. For example, a $Hex_5GlcNAc_2$ structure was found to carry up to 3-*O*-Me groups, one each on the terminal Hex. High degree of *O*-methylation may in effect be similar in certain aspects to multifucosylation. It is possible that due to evolutionary adaptation, *S. mansoni* have evolved a biosynthetic mechanism of multifucosylation to mimic or counteract the multi-*O*-methylation of freshwater snails, their intermediate hosts. Structural studies on the egg glycans of *S. haematobium* have also been initiated and will be presented.

C9.5

Determination of diagnostic glycoconjugate markers in the urine of *Schistosoma mansoni* infected individuals

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Worms of the human parasite *Schistosoma mansoni* as well as their eggs secrete a large variety of glycoconjugates into the host's circulation. Antigenic glycoconjugates like the adult worm circulating antigens CCA and CAA, and a number of egg antigens can be detected in serum or urine of schistosomiasis patients by ELISA using monoclonal antibodies (MAbs) directed against specific carbohydrate epitopes. The immunogenic nature of the detected secretory components implies that immune complexes are formed that are prone to clearance in the infected host. In addition, these antigens are (partially) catabolised and do not necessarilly end up as MAb-detectable antigens in urine. This limits the quantitative use of such assays for the determination of the infection intensity. Therefore we are aiming to find novel, ideally non-immunogenic markers directly detectable in urine of schistosomiasis patients. To this end we have isolated urinary oligosaccharides by solid phase carbon extraction. The oligosaccharides were profiled by HPAEC-PAD and, after labeling with APTS, by capillary electrophoresis with very sensitive fluorescence detection. Sofar, three compounds have been identified to be present exclusively in "infected" urine and not in control urine (n=10 each). The isolated compounds are under investigation by MALDI-TOF and ESI-Ion-Trap MS. The structural characteristics of the oligosaccharides and their relation to schistosomal infection will be described.

C9.6

Reactivity of MEST-1 (anti-gal*f*) with *Trypanosoma cruzi*. Immunolocalization of GIPCs in acidic vesicles of epimastigotes

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The reactivity of MEST-1 monoclonal antibody (anti terminal β-D-galactofuranose [1]) with *Trypanosoma cruzi* was analyzed using confocal microscopy. Positive immunofluorescence was observed with MEST-1 with different *T. cruzi* forms, except with intracellular trypomastigotes. Co-localization of MEST-1 and acidic vesicles was observed after incubation of epimastigotes with Lysotracker red™, a specific fluorescence marker for acidic organelles. Parasite delipidation abolished MEST-1 reactivity, indicating that the major antigens recognized by MEST-1 are present in the lipid fraction. Glycosylinositol phosphorylceramides (GIPCs) were extracted from epimastigotes, tissue-derived extracellular amastigotes and trypomastigotes and analyzed by high performance thin layer chromatography (HPTLC). Although the expression of GIPC amounts vary among the different *T. cruzi* forms (epimastigotes > amastigotes > trypomastigotes), it was verified by HPTLC immunostaining using MEST-1 that all GIPCs present terminal residues of gal*f*. (Supported by: FAPESP, CNPq, PRONEX).

Reference

Suzuki E *et al* (1997) *Glycobiology* **7**:463-468

C9.7

Schistosome glycoproteomics: characterisation of soluble egg antigens

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Eggs from schistosomes, human blood flukes that infect over 200 million people in the tropics, produce a complex mixture of glycoconjugate antigens which elicit a strong antibody response and play an important role in the main pathology of the disease. A large number of monoclonal antibodies have been raised against schistosome carbohydrate epitopes. Circulating soluble egg antigens (SEA) can be detected in urine or serum of schistosome-infected people making use of two of these monoclonal antibodies (Mabs): Mab 114-5B1 which recognises GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc and Mab 114-4D12 for which the precise epitope is still unknown [1,2]. In view of the diagnostic potential as well as to be able to understand the complex immunomodulatory effects of SEA a study was undertaken to identify those egg antigens that are detected with the mentioned Mabs and to characterise their glycosylation. The mixture of antigens isolated using affinity chromatography with Mabs 114-4D12 and 114-5B1 was studied by a combination of 2D-gel electrophoresis, Mab and lectin blotting, and mass spectrometry. The characterisation of the glycosylation of the egg antigens as well as their protein backbone will be presented.

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C9.8

Cloning of a *Toxoplasma gondii* UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase (ppGalNAc-T)

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Toxoplasma gondii is an intracellular protozoan parasite and an important human pathogen. To investigate the role of O-glycosylation in *T. gondii* biology, we cloned a putative UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase. Degenerate oligonucleotide primers yielded a specific ~250 bp PCR amplification product from *T. gondii* cDNA. This probe was used to identify genomic and cDNA clones from *T. gondii* λ-DashII and λ-ZapII libraries. Southern blotting confirmed that these clones are derived from parasite rather than the human DNA. Conceptual translation of the 751 amino acid protein predicts a type II membrane protein with a large extracytoplasmic C-terminal domain. As a result of comparison of cDNA and genomic DNA sequences, we determined that this protein is encoded by 10 exons in a 10 kb region. Expression of a YFP-ppGalNAc-T chimera in T. gondii demonstrated localization of this protein in the Golgi. The predicted catalytic domain was transiently expressed in COS7 cells as a secreted protein and purified from conditioned medium by immunoprecipitation using anti-FLAG beads. Further studies are currently underway to identify enzymatic activity for this putative protozoan ppGalNAc-T. In addition, we are designing a targeting construct to prepare genomic knockout organisms.

C9.9

Modelling studies on the effects of N-glycosylation on the conformation of surface proteins of *Trypanosoma brucei*

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Trypanosoma brucei, the causative agent of African sleeping sickness, is protected during the mammalian phase of its life cycle by a dense layer of VSG (variant surface glycoprotein) on its plasma membrane. This VSG can undergo antigenic variation thus rendering antibodies redundant. These proteins all have different amino acid, GPI anchor and N-linked glycan structures. However the partial crystal structures of the 2 VSGs suggest they are folded in a similar conformations. The type III VSG (MITat 1.5) has 3 potential N-link sites and the glycans are a mixture of 14 different structures. It was not known which of the 3 glycosylation sites were occupied. Using a combination of tryptic mapping, microbore HPLC and MALDI-TOF, we were able to define the range of glycoforms for this VSG. We found all three sites were occupied and the structures of the glycopeptides were elucidated. Using 3 dimensional modelling we built a structure of the VSG dimer based on the available partial crystal structures. The glycosylation sites are predicted to be clustered around the base of the N-terminal domain of the molecule.

References

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C9.10

Studies on the anthelmintic effects of inhibitors of the glycosphingolipid biosynthesis and choline metabolism

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Phosphorylcholine (PC) is a widespread antigenic eptitope of pathogens like parasitic nematodes, which are characterized by the presence of PC-substituted glycosphingolipids and proteins [1]. There is evidence that such PC-substituted molecules express multiple immunological effects by influencing signal transduction pathways in B- and T-lymphocytes [2]. *Caenorhabditis elegans*, a free-living nematode expressing analogous PC-substituted antigens [3], represents a suitable model for biosynthetic studies on this epitope due due to ist easy handling *in vitro* and fully sequenced genome. Cultivation of *C. elegans* in a chemically defined medium in the presence of various inhibitors of glycosphingolipid biosynthesis and choline metabolism displayed pronounced effects on the development of the worms and fertility. Such inhibitors might, therefore, be regarded as potential candidates for the development of anthelmintics.

References

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[3] Gerdt S *et al* (1999) *Eur J Biochem* **266**:952-963

C9.11

Role of linkage specific 9-*O*-acetylated sialoglycoconjugates in activation of the alternate complement pathway in mammalian erythrocytes

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Sialic acids play a critical role in controlling the life span of erythrocytes. A varied expression of linkage specific 9-*O*-acetylated sialoglycoconjugates as defined by the lectinogenic epitope of Achatinin-H namely 9-*O*-acetylated sialic acid α2→6 GalNAc was identified on mammalian erythrocytes. Although fluorometric quantitation of the percentage of (8)9-*O*-acetylated sialic acid on mammalian erythrocytes showed minor variations ranging from 20-25% for rabbit, guinea pig and hamster, Achatinin-H mediated agglutination showed wide variations being 1024, 32 and 32 units respectively. Percentage of linkage independent (8)9-*O*-sialic acids on rat and murine erythrocytes was high being 40 and 60% respectively, while Achatinin-H mediated agglutination was only 16 and zero units respectively. Linkage specific 9-*O*-acetylated glycotopes strongly correlated with their susceptibility towards alternate complement mediated lysis (r=0.9), not with their total 9-*O*-acetyl-sialic acid content (r=0.28). 9-*O*-acetylated sialic acid glycotopes have been identified on human erythrocytes of patients with *Visceral leishmaniasis* and were seen to possess a significantly enhanced degree of alternate complement mediated hemolysis. Our results indicate that complement mediated hemolysis depends not simply upon the extent of surface 9-*O*-acetylated sialic acids present but more importantly upon the specific linkage.

C10. Glycobiology of plants

C10.1

Triterpene glycosides from *Hedera colchica* leaves

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Hedera colchica C.Koch (Araliaceae) is widely used in traditional medicine. Our preliminary search of this plant showed extremely large total extent of triterpene glycosides and their very complicated composition. However, only few main glycosides were isolated earlier from the leaves of this plant. Isolation of triterpene glycosides from *Hedera colchica* leaves was carried out in usual maner by ethanol extraction followed by chromatographic separation on SiO_2 column with chloroform-ethanol-water gradient elution. Side by side with main known saponins namely 3-*O*-[β-D-glucopyranosyl-(1→4)]-[α-L-rhamnopyranosyl-(1→2)]-α-L-arabinopyranosides of oleanolic acid and hederagenin and their 28-*O*-α-L-rhamnopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-*O*-β-D-glucopyranosyl esters were isolated different degraded forms of these glycosides wich characterized by absence of one terminal sugar moiety (glucose or rhamnose) at C-3 carbohydrate chain and/or by absence of one (rhamnose) or two (rhamnose and glucose) terminal sugars in C-28 carbohydrate chain. It was found that in some minor glycosides α-L-arabinopyranosyl residue substituted with β-D-xylopyranosyl residue and some others minor glycosides posess an additional α-L-rhamnopyranosyl residue linked to C-4 hydroxyl group of terminal glucose in carbohydrate chain at C-3 aglycone atom. The structures of isolated glycosides were supposed by chemical search and different NMR-studies. Most of minor glycosides are new saponins.

C10.2

New and known triterpene glycosides from *Fatsia japonica* seeds

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Fatsia japonica Decne. et Planch. (Araliaceae) is a well-known ornamental plant from the Japan Islands. It is also used in traditional oriental medicine. Earlier we have studied the triterpene glycosides from leaves and fruit pericarp of this plant. Now we have carried out an investigation of *Fatsia japonica* seeds. Triterpene glycosides were extracted with 80% 2-propanol and separated by SiO_2 column chromatography with chloroform-ethanol-water as eluent. The following triterpene saponins were isolated: 3-*O*-β-D-glucopyranoside of hederagenin, 3-*O*-β-D-sophorosides of oleanolic acid and hederagenin, their 28-*O*-β-D-gentiobiosyl esters and the new 3-*O*-β-D-sophorosyl-28-*O*-α-L-rhamnopyranosyl-(1→4)-*O*-β-D-gentiobiosyl ester of hederagenin. We have detected all these glycosides in the seeds of different *Hedera* species. However, the glycoside composition of *Fatsia japonica* seeds is more complicated, and additionally containing 3-*O*-α-L-arabinopyranoside of hederagenin, 3-*O*-β-D-glucopyranosyl-(1→2)-*O*-α-L-arabinopyranoside of hederagenin, their 28-*O*-β-D-gentiobiosyl esters and some glycosides of hypsogenin: hypsogenin-3-*O*-β-D-sophoroside and its 28-*O*-β-D-gentiobiosyl ester. The last compound is a new glycoside. The structures of all isolated saponins were determined on the basis of chemical and various NMR methods.

C10.3

Four new triterpene glycosides from *Tetrapanax papyriferum* stems

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Tetrapanax papyriferum C.Koch, widespread in Taiwan and South China, belongs to the *Araliaceae* family, which plants are usually rich in triterpene glycosides. Only leaves and roots of this plant were analysed earlier. We have isolated four triterpene glycosides from the *Tetrapanax papyriferum* stems. Triterpene glycosides were extracted with 80% 2-propanol. Glycosides were fractionated by column chromatography on silicagel with chloroform-ethanol-water as eluent. Four individual pure compounds were obtained after chromatographic separation. Their complete structures were established on the basis of chemical methods (alkaline and acid hydrolysis), thin-layer chromatography identification of hydrolysis products and spectral data (different ^{1}H and ^{13}C-NMR experiments - COSY, HETCOSY and ROESY). The following structures were assigned to isolated glycosides: 3-*O*-[β-D-glucopyranosyl-(1→3)]-[β-D-galactopyranosyl-(1→2)]-*O*-α-L-arabinopyranosides of oleanolic and echinocystic acids and their 28-*O*-α-L-rhamnopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-*O*-β-D-glucopyranosyl esters. All of these substances are new saponins.

C10.4

Study of triterpene glycosides from *Cussonia paniculata* leaves

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Cussonia paniculata Eckl. et Zeyh., a plant of the Araliaceae family is widespread in Madagascar, and was not studied earlier. The leaves of this plant are very rich in triterpene glycosides. From the ethanol extract of the leaves, by chromatographic methods more then 20 individual glycosides were isolated in pure state. Among them are earlier known β-amyrin row glycosides, namely, 3-*O*-α-L-arabinopyranosides of oleanolic acid and hederagenin, 3-*O*-β-D-glucopyranosyl-(1→2)-*O*-α-L-arabinopyranosides of oleanolic acid and hederagenin and their 28-*O*-α-L-rhamnopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-*O*-β-D-glucopyranosyl esters. However, one of the main triterpene glycosides is a new glycoside of α-amyrin row - 28-*O*-α-L-rhamnopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-*O*-β-D-glucopyranosyl ester of 23-hydroxyurs-12-en-28-oic acid. The structure of this glycoside was determined on the basis of chemical and various NMR methods (^{1}H-, ^{13}C-NMR, COSY, HETCOSY, ROESY, HSQC and HMBC). It was found that monoacetyl derivatives of all these glycosides bearing an acetyl group in the 28-O-saccharide chain occur in *Cussonia paniculata* leaves. Determination of the exact location of the *O*-acetyl group is in progress. Minor glycosides of *Cussonia paniculata* leaves are presented by acidic glycosides of oleanolic acid containing D-glucuronic acid in a 3-O-saccharide chain.

C10.5

Oligochitin elicitor-binding protein from plasma membrane of rice cells

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N-Acetylchitooligosaccharides ($GlcNAc_6$) could induce the formation of phytoalexin in suspension-cultured rice cells. High affinity binding site for this elicitor was detected in the plasma membrane of rice cells and a corresponding binding protein was identified by affinity labeling. In the present study, we report the purification of this elicitor-binding protein (EBP) from the plasma membrane (PM) by affinity chromatography using a newly designed affinity matrix and survey its gene. The detergent solubilized PM was applied to a $GlcNAc_8$-APEA-CH-Sepharose column, which was then washed with buffer and several elicitor-inactive sugar solutions. The bound fraction was eluted with acidl buffer (pH 2.3) and immediately neutralized with 1 M Tris solution. The purified protein showed the specific binding activity to ^{125}I-labeled $GlcNAc_8$-APEA derivative as proved by the affinity crosslinking with glutaraldehyde. SDS-PAGE followed by silver-staining as well as affinity labeling showed the presence of two protein bands, corresponding to 75 and 55 KDa. The result suggested that EBP was cleaved with protease during purification. The bands detected by the affinity labeling disappeared by the addition of the unlabeled elicitor active sugar. The recovery of EBP obtained by the use of the new affinity matrix was approximately 18 times better than that by $GlcNAc_7$-Lys-Sepharose. The increased recovery of EBP paved the way for the analysis of the N-terminal amino acid sequence. The survey of this EBP gene using a probe corresponding to the N-terminal amino acid sequence of EBP is in progress.

C10.6

Isolation and characterization of rice genes responsive to *N*-acetylchitooligosaccharide elicitor by DNA microarray

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N-acetylchitooligosaccharides are potent elicitors to suspension-cultured rice cells, inducing a set of defense reactions such as change of ion-fluxes, production of reactive oxygen species and phytoalexins etc. Expression of defense-related genes is considered to play important roles in the defense reactions, and we have isolated and characterized elicitor-responsive genes and elucidated the relationship between the early events such as cytoplasmic acidification or reactive oxygen species and gene expression. In order to get further insight, we carried out DNA microarray analysis to identify more numbers of elicitor-responsive genes. Some detailed analysis of the expression of these newly isolated genes will be reported.

Reference

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C10.7

Cloning and expression of cDNAs encoding α1,3-fucosyltransferase homologues from plants

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We report the identification, isolation and characterisation of the cDNAs of three genes (FucTA, FucTB and FucTC) encoding proteins similar to α1,3-fucosyltransferases in *Arabidopsis thaliana*. RT-PCR was used to amplify the full length coding sequence of FucTA. The FucTA gene encodes a presumptive protein of 501 amino acids showing an overall sequence identity of 66% to the protein encoded by the recently isolated mung bean Fuc-T C3 cDNA. FucTA was expressed in *Pichia pastoris* and found to catalyse the same reaction as mung bean core α1,3-fucosyltransferase as judged by analyses of the products by MALDI-TOF and HPLC. The FucTB cDNA used an alternative splicing site between the second and third exon resulting in a premature stop codon. The FucTC gene encodes a protein with less than 40% identity to FucTA across 115 amino acids of a total of 401 amino acids and is a member of a new sub-family of plant α1,3/4-fucosyltransferase homologues. Whereas *Arabidopsis* FucTC was seemingly inactive, tomato FucTC (identified by searching of the EST database) was found to be active as a Lewis-type α1,4-fucosyltransferase and so is a candidate for the enzyme that generates Lewis-a epitopes on N-glycans in plants.

C11. Glycobiology of viruses

C11.1

GM3-Replica peptides which inhibit influenza virus infection

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Influenza virus A is the causative agent of a serious disease in humans for a long time. There has been little success in the development of effective anti-influenza virus agent, because of the capacity of the virus to modify its surface antigens, hemagglutinin (HA) and sialidase (NA), leaving the immune system unable to cope with essentially new antigens. Influenza HA is known to bind sialylgalactoside having α(2-3) or α(2-6) linkages of receptors on host cells in the first step of infection.

The phage displayed pentadecapeptide library was used to obtained the peptides which can bind HA. HA1 and HA3 were extracted with ether from Influenza virus A/PR/8/34 and A/Victoria/1/75, respect-ively. The HA-specific phages were collected by the elution with GM3.

The specificity of the cloned phages were determined by ELISA method. Futhermore, infection of Influenza virus to MDCK cells were determined by LDH assay. The liposomes (eggPC/cholesterol = 2/1 by mol) containing 10 mol% lipopeptide inhibited the infection of Influenza virus to MDCK cells. The inhibition efficiencies were almost same with these of sialyl Le^x-replica peptides.

In this report, we demonstrated that selection of HA-binding peptide from phage library is a new strategy for the development of anti-viral agent.

C12. Glycobiology of yeasts, fungi and bacteria

C12.1

The diversity of immunosuppressive activity of bacterial lipopolysaccharide

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Connected with virulence the ability of bacterial lipopolysaccharide (LPS) to suppress cell-mediated immune respons had been studied. LPS of *Shigellae, Salmonellae, Klebsiellae* and *Pseudomonas* were extracted by the water-phenolic mixture or the immunosorbents from antibodies to O-antigens of the bacteria. In the study delayed type hypersensitivity (DTH) to test-antigen (footpad method) in mice was used. Three immunosuppressive components were found in native or redox activated LPS by the Sephadex G-200 gel filtration. These were components with molecular weight I) about 800 kD and higher, II) about 150-200 kD and III) about 50-70 kD. A quantity of active and inactive components in LPS was changed, it was depended on level of strain virulence. All of III components of *Pseudomonas, Shigellae, Salmonellae* and *Klebsiellae* possessed the same immunosuppressive properties. They were sensitive to the heating and expressed inductive immunosuppressive action on DTH. II and also III LPS components of these bacteria significantly differ from other one by heating and nature of immunosuppressive action. That differences were found also directly between II and III components of LPS. According to preliminary investigation the peculiarity of immunosuppressive action first of all depended on structure of components.

C12.2

Antibodies to ceramide monohexosides from *Cryptococcus neoformans* and *Pseudallescheria boydii* inhibit fungal growth and differentiation

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Ceramide monohexosides (CMHs) were purified from lipidic extracts of the fungal pathogens *Cryptococcus neoformans* and *Pseudallescheria boydii*. By using a combination of physical and chemical techniques, CMHs from these species were characterized as glucosylceramides, with the carbohydrate residue attached to 9-methyl-4,8-sphingadienine in amidic linkage to 2-hydroxyoctadecanoic or 2-hydroxyhexadecanoic acid. Sera from individuals with cryptococcosis, pseudallescheriasis or other mycoses recognized these molecules. Specific antibodies, which were purified from sera, bound to *C. neoformans* yeasts, as well as to mycelial and pseudohyphal, but not conidial forms of *P. boydii*. Confocal and electron microscopy followed by chemical analysis showed that the cryptococcal CMH is predominantly located at the fungal cell wall. The addition of antibodies to CMH to cultures of *C. neoformans* inhibited cell budding and growth. In *P. boydii*, the presence of antibodies to CMH inhibited the conversion of conidial to mycelial forms. These results suggest therefore that CMHs are cell wall antigens linked with crucial events of the fungal biology, such as cell growth and differentiation.

C12.3

Immunochemical analysis of *Citrobacter* serogroup O2 and structure of the O-specific polysaccharide of the lipopolysaccharide from *C. youngae* O2a,1b

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Bacteria of the genus *Citrobacter* are inhabitants of the intestinal tract and may cause gastroenteritis. The genus is divided into 11 species and 43 O-serogroups. However, some O-antigens in different species are related, and strains of some serogroups are serologically heterogeneous. We report here the structure of the O-specific polysaccharide of *Citrobacter youngae* serovar O2a,1b:5,6; strain PCM 1507. The O-polysaccharide of LPS was studied by sugar and methylation analyses and ^{1}H- and ^{13}C-NMR spectroscopy, including two-dimensional ^{1}H,^{1}H COSY, TOCSY, NOESY, and ^{1}H,^{13}C HSQC experiments. The structure of the repeating unit of the polysaccharide was established:

α-D-Xyl*p*-(1→4)
/
→4)-α-D-Rha*p*-(1→3)-β-D-Man*p*-(1→4)-β-D-Rha*p*-(1→

Serological studies showed that among 5 strains formerly assigned to *Citrobacter* serogroup O2, three strains, PCM 1507, PCM 1494 and PCM 1496, have an S-type LPS reactive with specific anti-O2 serum, and thus do belong to the serogroup O2. Strain PCM 1495 was found to be R-mutant. Strain PCM 1573 has an S-type LPS but it does not react with anti-O2 serum, and thus this strain belongs to another serogroup.

C12.4

The Yta7p, member of the AAA protein family, is involved in the dolichol biosynthesis in the yeast *Saccharomyces cerevisiae*

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The Yta7p of *Saccharomyces cerevisiae*, which was isolated in our laboratory in two-hybrid screen as a putative protein factor interacting with farnesyl diphosphate synthase (FPPS), belongs to the AAA protein family (ATPases associated with divers cellular activities). Its function in yeast has not been documented previously. We have found that deletion of *YTA7* leads to hypersensitivity to lovastatin (inhibitor of HMG-CoA reductase – key enzyme of mevalonate pathway) indicating its possible involvement in isoprenoid biosynthesis regulation. Further experiments demonstrated a diminution of the specific activity of *cis*-prenyltransferase (first enzymes of dolichol branch of mevalonate pathway, encoded by *RER2 and SRT1* genes), corrected by exogenous farnesyl diphosphate addition, and elevated level of squalene (first intermediate of ergosterol metabolism) in *YTA7* deleted strain. Genetic and biochemical analysis of *RER2* or *SRT1* deleted strains indicates that Yta7p might be involved in Rer2p but not Srt1p function.

C12.5

A peptidorhamnomannan from the mycelium of *Pseudallescheria boydii* is a potential diagnostic antigen of this emerging human pathogen

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The ascomycete *Pseudallescheria boydii* is an emerging human pathogen frequently found in soil and polluted water. A peptidopolysaccharide antigen has been isolated from mycelial forms of *P. boydii*, and characterized using chemical and immunological methods. Monosaccharide composition, methylation analysis, and ^{1}H and ^{13}C-nuclear magnetic resonance spectra indicated the presence of a rhamnomannan with a structure distinct from those of similar components isolated from other fungi, containing Rha*p* (1→3) Rha*p* epitopes on side chains which may be linked (1→3) to (1→6)-linked mannose. The peptidorhamnomannan from *P. boydii* reacted poorly with an antiserum raised against whole cells of *Sporothrix schenckii* and strongly with that against *P. boydii* hyphae. Such characteristics and immunological differences make this major rhamnose-containing antigen of *P. boydii* a unique structure among pathogenic fungi, which can be used for the specific diagnosis of this fungus.

C12.6

Overexpression of GDP-mannose pyrophosphorylase in *Saccharomyces cerevisiae* corrects defects in dolichol-linked saccharide formation and protein glycosylation

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All mannose residues of yeast glycoconjugates are derived from GDP-Man or dolichol activated Man (DolP-Man). To assess the role of GDP-Man availability for the synthesis of dolichol-linked saccharides, we have used thermosensitive mutants from *Saccharomyces cerevisiae*, affected in asparagine-linked glycosylation: in DolP-Man synthase (*dpm1*), in β-1,4 mannosyltransferase (*alg1*) and in α-1,3 mannosyltransferse (*alg2*). The mutants were transformed with the yeast *MPG1* gene encoding GDP-Man pyrophosphorylase catalysing formation of GDP-Man. We found that overexpression of *MPG1* allows growth at the nonpermissive temperature, leads to up to three fold increase in the cellular content of GDP-Man and corrects various glycosylation defects ascribed to the mutants. Our date indicate that an increased level of GDP-Man is able to correct defects in mannosylation reactions localised in the endoplasmic reticulum and to the Golgi.

C12.7

Common carbohydrate determinants on the surface of Mollicutes and on the phylogenetically related bacteria from the *Bacillus-Lactobacillus-Streptococcus* group

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A number of monosaccharide constituents of the glycocalix carbohydrates of some Mollicutes (*Mycoplasma pneumoniae* FH, *M. hominis* PG21, *M. fermentans* PG18, *Acholeplasma laidlawii* PG8, *A. laidlawii* var. *granulum* 118) and of phylogenetically related bacteria from a *Bacillus-Lactobacillus-Streptococcus* group (*Bacillus cereus* 89, *B. cereus* DM423, *B. subtilis* 1/2, *B. licheniformis* 31, *B. licheniformis* 49, *Lactobacillus plantarum* 337D, *L. plantarum* 11/16, *Streptococcus thermophilus* S1, *S. thermophilus* S5, *Enterococcus faecium* K50) were recognized by electron microscopy by using plant lectins labeled with colloid gold. It is marked that for all microorganism cells the typical finding was the interaction with all used plant lectins while for the researched *Bacillus* no binding with the lectin from *Pisum sativum L.* was detected, which suggests the absence of α-D-glucose in the composition of the *Bacillus* surface glycopolymers. The common carbohydrate determinants for the microorganisms glycocalix from related groups were: sialic acid, L-fucose, α- and β-D-galactose, α-*N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, and α-D-mannose. This characteristics confirms the common origin of the mentioned microorganisms and determine their biological activity.

C12.8

Protein *O*-mannosyltransferase from the filamentous fungus *Trichoderma reesei*; isolation of c-DNA and characterisation

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Protein *O*-mannosyltransferase (PMTp) initiates synthesis of O-glycosidic linkage by the transfer of mannose from dolichyl activated sugar to seryl or threonyl residues of secretory proteins. Activity of the enzyme is up-regulated in the hypersecretory strains of *T. reesei.* On the other hand a large number of intrinsic secretory proteins of *T. reesei* are *O*-mannosylated. To asses the role of *O*-mannosylation in protein secretion we have isolated from *Trichoderma* lambda ZAP cDNA library a cDNA fragment encoding putative PMTp containing 769 AA. Predicted protein sequence shows 51% identity to *S. cerevisioae* Pmt4p, 53% identity to *S. pombe* and 33% to human *pmt1* enzymes. *T. reesei* PMT4p contains 10 transmembrane domains located symmetrically at the N- and COOH- terminus. Northern analysis indicates that mRNA level for PMT4p relates to the secretory capacity of various *Trichoderma* strains.

C12.9

β-Galactofuranose-containing O-linked oligosaccharides present in the cell wall peptidogalactomannan of *Aspergillus fumigatus* bear immunodominant epitopes

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O-linked oligosaccharides ranging from di- to hexasaccharide were β-eliminated from the peptidogalactomannan (pGM) of *Aspergillus fumigatus* mycelial cell wall by reductive mild alkaline treatment. Their primary structures were deduced based on analysis by GLC, ESI-QTOF-MS), ^{1}H-1D and ^{1}H-^{13}C correlation 2-D NMR as being: α-Glc*p*-(1→6)-Man-ol, β-Gal*f*-(1→6)-α-Man*p*-(1→2)-Man-ol, β-Gal*f*-(1→5)-β-Gal*f*-(1→6)-α-Man*p*-(1→2)-Man-ol and β-Gal*f*-(1→5)-(β-Gal*f*-(1→5))$_3$-β-Gal*f*-(1→6)-Man-ol. The β-Gal*f* bearing oligosaccharides have never been described as fungal O-linked glycans.
The pGM is antigenic [1] and was recognized by human sera of patients with aspergilose when probed in an ELISA experiment, but its de-O-glycosylation rendered a 50% decrease in the reactivity. Furthermore, when tested in a hapten inhibition test, the isolated oligosaccharide-alditols were able to block in a dosage-response basis the recognition between human sera and the intact pGM. The immunodominant epitopes were present in the tetra- and hexasaccharides, which contain the motif β-Gal*f*-(1→5)-β-Gal*f* at the non reducing terminal.

Reference

[1] Haido RMT *et al* (1998) *Medical Mycology* **36**:313-321

C12.10

Overexpression of the genes involved in early steps of protein O-mannosylation in *Trichoderma reesei*; Effect on protein glycosylation and secretion

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Overexpression of the *S. cerevisiae DPM1* gene, encoding dolichol phosphate mannose synthase (DPMS), in *T. reesei,* resulted in increased protein secretion and abnormal cell wall structure [1]. Simultaneously, we have demonstrated that in the yeast *S. cerevisiae* the cellular level of GDPMan plays a regulatory role at least in the early steps of protein glycosylation (Janik *et al*, in preparation). Thus, to find possible link between glycosylation and secretion, we have cloned *T. reesei* genes involved in the final reaction of GDPMan synthesis [*mpg1*, encoding GDPMan pyrophosphorylase (GDPMP)] and *dpm1*, encoding DPMS, and overexpressed them in *T. reesei.* There was no effect on the rate of protein secretion. Overexpression of *mpg1* however, lead to the extension of the O-linked mannose chains of the secreted enzymes and to the changes of their catalytic properties. Moreover, transcription of the *dpm1* was increased significantly in the strains bearing an additional copy of *mpg1* gene, indicating that in *T. reesei* the cellular level of GDPMan might be the rate limiting factor in protein O-mannosylation.

Reference

[1] Kruszewska JS *et al* (1999) *Appl Environ Microbiol* **65:**2382-2387

C12.11

Structural studies of an exopolysaccharide produced by an *Alteromonas* strain isolated from hydrothermal deep-sea vents

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An *Alteromonas* strain was recently isolated from deep-sea hydrothermal vents and called *Alteromonas infernul* [1]. This new marine bacterium is able to produce, in large quantities (8 g/L), a complex exopolysaccharide (EPS) that displays strong anticoagulant properties after sulfation and partial depolymerization [2]. In order to establish "structure-activity" relationships, the structural elucidation of the native polysaccharide has been undertaken. These studies were not easy, for three reasons: (1) the EPS is indeed constituted of two polymers impossible to separate, (2) NMR spectra cannot be recorded even with an HRMAS equipment, (3) partial hydrolysis gave an heterogeneous mixture, because the major component is highly branched. Nevertheless, a purified fraction was obtained by chromato-graphy, its structure (constituted of 11 different residues) was established from methylation analysis and NMR data.

References

[1] Raguenes GH *et al* (1997) *J Appl Microbiol* **82:**422-430
[2] Guezennec J *et al* (1998) *Carbohydr Polym* **37:**19-24

C12.12

Modification of the N-glycosylation pattern of fungi for the humanization of recombinant therapeutic proteins

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Fungal species are frequently used for the production of recombinant proteins. However, in contrast to mammals, they mostly synthesize high-mannose type N-glycans. This hampers the therapeutic use in men, of glycoproteins synthesized by these organisms. As such, we wish to modify the fungal N-glycosylation biosynthesis pathway towards the synthesis of human-compatible N-glycan structures.
The predominant N-glycans present on the CBHI of *Trichoderma reesei* RUTC30 were analysed. Only a small amount of mammalian-like high-mannose N-glycans ($Man_{5-7}GlcNAc_2$) was found. However, after overexpression of the human N-acetylglucosaminyltransferase and NMR analysis of the N-glycans present on CBHI, we could demonstrate the conversion of $Man_5GlcNAc_2$ to $GlcNAcMan_5GlcNAc_2$.
Only a limited amount of the *Trichoderma* N-glycans could be trimmed to $Man_5GlcNAc_2$ by α1,2-mannosidase, both *in vivo* and *in vitro*. This was due to the presence of capping residues such as α1,3-linked glucose residues. This points out to an incomplete processing of the ER precursor $Glc_3Man_9GlcNAc_2$ by glucosidase II. Cloning of the gene clearly indicated the presence of a frame shift, presumably leading to the synthesis of a partially inactive glucosidase II.

C12.13

Cloning and expression of *Helicobacter pylori* GDP-L-fucose synthesizing enzymes (GMD and GMER) in *Saccharomyces cerevisiae*

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Helicobacter pylori is a Gram-negative gastric pathogen causing diseases from mild gastric infections to gastric cancer. The difference in clinical outcome is thought to be due to strain differences. *H. pylori* undergoes phase variation by changing its LPS structure according to the environmental conditions. The O-antigen of *H. pylori* contains fucosylated glycans, similar to Lewis structures found in human gastric epithelium. These Lewis glycans of *H. pylori* have been suggested to play a role in pathogenesis in the adhesion of the bacterium to gastric epithelium. In the synthesis of fucosylated structures, GDP-L-fucose is needed as a fucose donor. Here, we cloned two key enzymes of GDP-L-fucose synthesis, *H. pylori gmd* coding for GDP-D-mannose dehydratase (GMD), and *gmer* coding for GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase/4-reductase (GMER) and expressed them in an enzymatically active form in *Saccharomyces cerevisiae.* We also used the end product of these enzymes, GDP-L-fucose, as a fucose donor in a fucosyltransferase assay converting sialyl-*N*-acetyllactos-amine (sLN) to sialyl Lewis x (sLex).

C12.14

Evidence that in fission yeast cell wall α-(1→3)-glucan chains are interconnected extracellularly via an α-(1→4) linker

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The fungal cell wall is essential for cell viability, because fungal cells will lyse without a cell wall. α-Glucan is a major cell wall constituent and is believed to contribute in the mechanical strength and rigidity of the cell. Recently, we identified a putative α-glucan synthase Ags1p in fission yeast *Schizosaccharomyces pombe*. The protein was proposed to consist of three domains, namely an intracellular synthase domain, a pore forming multipass transmembrane domain, and an extracellular domain that might function as a transglycosylase. A point mutation in the extracellular domain resulted in a weakened cell wall, leading to loss of the characteristic rod-like cell shape and to cell lysis.
Here, we report the chemical structures of wild type and mutant α-glucan. Using HPSEC, it was shown that the average degree of polymerization (DP) of wt α-glucan is approximately 260 glucose units. Periodate treatment resulted in a 50% decrease of the DP without substantial change in polydispersity. Remarkably, mutant α-glucan before and after periodate oxidation both have DPs of approximately 125 residues. These data suggest that two discrete linear (1→3) linked glucose chains are interconnected via a number of (1→4) linked glucose residues, probably involving the extracellular domain of Ags1. The data obtained in this study will contribute to our understanding of the biosynthesis of the fungal cell wall.

C12.15

Structural elucidation of glycosylinositol phosphorylceramides from yeast and mycelium forms of *Sporothrix schenckii*

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Glycosylinositol phosphorylceramides (GIPCs) were extracted from mycelium and yeast forms of the thermally dimorphic mycopathogen *Sporothrix schenckii*. Three fractions from the mycelium form (Ss-M1, -M2, -M3), and two minor and the major fraction from the yeast form (Ss-Y1, -Y2, and -Y6, respectively) were isolated and their structures elucidated by 1- and 2-D ^{13}C- and ^{1}H-NMR, ESI/MS, and component analysis by GC/MS. The structures of Ss-M1 and Ss-M2 were determined to be Manα1→6Ins1-P-1Cer and Manα1→3Manα1→6-Ins1-P-1Cer, respectively. Ss-Y6 was determined to be a GIPC with a novel glycan structure, Manα1→3Manα1→6GlcNH$_2$α1→2Ins1-P-1-Cer. While the GlcNH$_2$α1→6Ins1-P- motif is found widely distributed in eukaryotic GPI anchors, the linkage GlcNH$_2$α1→2Ins1-P- has not been previously observed in any glycolipid. Ss-Y1 and Ss-Y2 were both found to have the known glycan structure Manα1→3Manα1→2Ins1-P-1Cer. These results demonstrate that *S. schenckii* can synthesize GIPCs with three different core linkages: (i) Manα1→2Ins, (ii) Manα1→6Ins, and (iii) GlcNH$_2$α1→2Ins. (Supported by: FAPESP, CNPq, PRONEX and NIH).

C12.16

Modification of the N-glycosylation pathway of *Aspergillus niger* by heterologous expression of α-1,2-mannosidase and *N*-acetylglucosaminyltransferase I

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A problem that arises when using fungi as hosts for the production of therapeutic proteins is that fungi only synthesise N-glycans of the high mannose type whereas humans have complex type N-glycans. Therapeutic proteins carrying these high mannose structures are rapidly cleared from the blood stream.
We started a strategy to humanise the N-glycosylation pathway in the filamentous fungus *Aspergillus niger* NW195. In a first step a HDEL tagged α-1,2-mannosidase from *Trichoderma reesei* was introduced. The over-expression of this enzyme leads to the conversion of the majority of the N-glycans to Man$_5$GlcNAc$_2$ (ca. 80%). In a second step the over-expression of human *N*-acetylglucosaminyltransferase I leads to the generation of the GlcNAcMan$_5$GlcNAc$_2$ structure. The percentage conversion of Man$_5$GlcNAc$_2$ to GlcNAcMan$_5$GlcNAc$_2$ was inversely related to the amount of protein that was synthesised, varying from ca. 40% to almost 100%.

C12.17

The yeast *SEC20* gene is required for N- and O-glycosylation in the Golgi: Evidence that impaired glycosylation does not correlate with the secretory defect

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The Golgi plays a fundamental role in posttranslational glycosylation, transport and sorting of proteins. The mechanism of protein transport through the Golgi has been seen controversial in recent years. During the characterization of N-glycosylation defective mutants (*ngd*) previously isolated by this laboratory, it was found that *ngd20* is allelic to *sec20*. *SEC20* was reported to be required for transport from ER to Golgi, but its precise function remains to be determined. We show now that *SEC20* is also required for N- and O-glycosylation in the Golgi, but not in the ER, in a cargo specific manner, and that the glycosylation defect does not correlate with the secretory defect. By pulse-chase labeling experiments in combination with mannose linkage specific antibodies, invertase and carboxypeptidase were found to be efficiently secreted to their final compartment, even upon shift to the non-permissive temperature, while glycosylation in the Golgi was severely impaired. Using microsomal membranes isolated from *ngd20* we find that mannosyl transfer from GDP-Man to various mannose-oligosaccharides, indicative for Golgi mannosylation, is strongly diminished. Analysis of the carbohydrate component of chitinase, an exclusively O-mannosylated protein, or of the bulk mannoprotein indicates that O-mannosylation is also reduced. The results demonstrate that *SEC20* affects in addition to secretion also glycosylation in the Golgi, presumably due to exerting a more general role in maintainance and function of the Golgi compartments.

C12.18

Effect of plant lectins on *Ustilago maydis in vitro*

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U. maydis is an edible parasitic basidiomycete that affects specifically corn (*Zea mays*) and Teocinte (*Z. diploperennis*). To identify the interaction between the basidiomycete and its host organism we tested the effect of plant lectins with well-known sugar specificity on growth and germination of *U. maydis* spores. Our results indicate that lectins specific for GalNAc, such as *Dolichos biflorus* and *Phaseolus lunatus*, and the wheat germ agglutinin specific for GlcNAc, inhibit spore germination, but are ineffective in modifying *Ustilago* cell growth [1]. The galactose-specific lectin from the corn coleoptile inhibited both germination and cell growth; whereas the lectin Concanavalin A (Man/Glc-specific) activated spore germination and growth. *Ulex europaeus* (L-Fuc specific) was ineffective to modify either growth or germination of spores. Our results suggest that specific saccharide-containing receptors participate in the regulation of growth and germination of *U. maydis* spores. (Financed in part by CONACyT and PAPIIT-UNAM).

Reference

[1] Santiago AP *et al* (2000) *Cell Mol Life Sci* **57**:1986-1989

C12.19

O-Mannosylation is essential for maturation, stability and function of cell wall stress sensors in *Saccharomyces cerevisiae*

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Protein O-mannosylation is an essential protein modification in yeast [1]. Furthermore, it is indispensable for cell morphology and cell wall integrity. Yet, how O-mannosylation affects cell wall architecture is still obscure.
Characterizing the O-mannosylation mutant *pmt2pmt4* we found that the mutant phenotype closely resembles mutants in the cell wall integrity signaling (PKC1-) pathway. The PKC1-pathway monitors cell wall stability and loss of any of its components results in cell lysis defect that is attributable to a deficiency in cell wall construction [2].
Overexpression of components of the PKC1-pathway suppress the *pmt2pmt4* cell wall defects. Further analysis of the MAPK-pathway activated by PKC1 revealed that O-mannosylation is essential for induction of the PKC1-pathway upon external stresses. Biochemical analysis of the highly O-mannosylated upstream receptors of the cell wall integrity pathway showed that abated O-mannosylation affects their maturation, stability and function.

References

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C12.20

Sphingosine-containing endotoxin from glucose-non fermenting Gram-negative clinical isolate induces uveitis (EIU) in rats which is not inhibited by polymyxin B

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A glucose-nonfermenting Gram-negative bacterial strain was isolated from bronchofiberoscope used for examination of patients suffering from pulmonary diseases. Lipopolysaccharide (LPS) isolated from bacterial mass contained components typical for LPS and also sphingosine, a component rarely occurring in bacteria. It was thus important to investigate the biological activity of endotoxin from this clinical isolate. Endotoxin-induced uveitis (EIU) was induced in rats by a single foot pad injection of LPS and the ocular inflammation has been observed after 24 h. The LPS isolated from clinical strain was two times more active in inducing EIU than LPS from *Shigella sonnei* Phase I and *Escherichia coli* K12 strains. The polymyxin B (PmB) did not inhibit the EIU induced by this lipopolysaccharide in contrast to the complete inhibition by PmB of *Escherichia coli* K12 LPS activity. Results indicate that LPS from studied clinical isolate has potent endotoxic activity. The structural difference of lipid A in LPS containing sphingosine may be responsible for the lack of inhibition effect of polymyxin B on induction of EIU.

Reference

Mieszala M *et al* (1997) Acta Biochem Polon **44:**293-300

C12.21

Fatty acid composition of lipid A of *Ralstonia solanacearum* lipopolysaccharides

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Lipopolysaccharides (LPS) isolated from four strains of *Ralstonia solanacearum* ICMP 5712, 6524, 8115 and 8169 were treated with 1% acetic acid 100°C for 2,0 h to precipitate lipid A. Methyl esters of fatty acids were analyzed by gas-liquid chromatography/mass spectrometry equipped by computer assistance. In all strains tested lipid A contained: (a) saturated fatty acids – dodecanoic ($C_{12:0}$), tetradecanoic ($C_{14:0}$), hexadecanoic ($C_{16:0}$) and octadecanoic ($C_{18:0}$) (exept of strain 6524); (b) hydroxylated – 3-OH- and 2-OH-hexadecanoic acids; the differences between the strains were observed on the amount of these acids: from 27.9% up to 35.5% (3-OH-$C_{16:0}$) and from 6.8% up to 24.2% (2-OH-$C_{16:0}$) in dependence on the strain tested; (c) two unsaturated fatty acids were found: $C_{16:1}$ (1.7%) presented in the only 8169 strain, while $C_{20:2}$ was detected in two strains 5712 (9.0%) and 8115 (31.8%). Some authors supposed that unsaturated fatty acids in lipid A resulted from dehydratation of hydroxylated fatty acids. The high amount of $C_{20:2}$ in *R. solanacearum* 8115 lipid A is not coincided with such suggestion. So far as 3-OH- and 2-OH-hexadecanoic and also tetra- and hexadecanoic acids are predominant in lipid A tested they are probably obligatory components which distinguish the representatives of *R. solanacearum* from others bacterial species.

C12.22

Filamentous fungus *Aspergillus oryzae* has two types of α-1,2-mannosidases, one of which is a microsomal enzyme that removes a single mannose residue from $Man_9GlcNAc_2$

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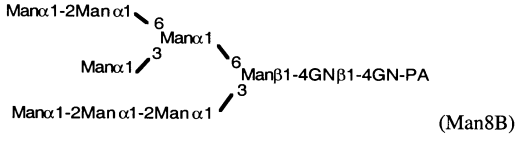
α-Mannosidase activities towards high-mannose oligosaccharides were examined with a detergent-solubilized microsomal preparation from a filamentous fungus, *Aspergillus oryzae*. In the enzymatic reaction, the pyridylaminated $Man_9GlcNAc_2$ was trimmed to Man8B, which remained predominant after a prolonged reaction. Trimming was optimal at pH 7.0 in the presence of Ca^{2+} and kifunensine was inhibitory with IC_{50} below 0.1 μM. These results suggest that the activity is the same type with human and yeast endoplasmic reticulum (ER) α-mannosidases. Considering the results together with our previous data on a fungal α-1,2-mannosidase that trimmed $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$, the filamentous fungi appear to have two types of α-1,2-mannosidases, each of which acts differently on N-linked mannooligosaccharides.



(Man8B)

C12.23

Immunobiologic properties of chemically modified *Shigella* and *Salmonella* lipopolysaccharide

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Influence of *Shigella sonnei* and *Salmonella typhimurium* lipopolysaccharide (LPS) on antibody formation and expression of delayed type hypersensitivity (DTH) to several antigens in mice had been studied. It was used LPS extracted by water-phenolic mixture (LPS-PW) and also this LPS after redox treatment by 2-mercaptoethanol (LPS-RD). It was found that i.p. injection of LPS-RD (1 mkg) inhibited DTH to non-bacterial antigen and injection of LPS-PW did not influence on the DTH level. In this case *S. typhimurium* LPS-RD turned out direct immunosuppressive action on DTH and *S. sonnei* LPS-RD expressed inductive immunosuppressive action. The last one inhibited DTH, if was injected in mice together with avirulent *S. sonnei* strain. In the case of immunization by LPS-RD the mice replied by more intensive antibody formation to O-antigen, than in the case of immunization by LPS-PW. If LPS-PW or LPS-RD were injected together with sheep erythrocytes antibody level to erythrocytes was higher in the case of LPS-RD injection. Thus, LPS-RD combines ability to suppress cell-determined immune response with its high immunogenic and immunostimulating activities in the humoral immune system. Such properties are characteristic of native LPS of virulent *S. sonnei* strains (LPS-V). Probably, *Shigella* and *Salmonella* LPS-RD are equivalents of their LPS-V.

C12.24

Structural polysaccharides of the cell wall of *Aspergillus fumigatus*

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The cell wall of human pathogen plays an essential role in the host-fungus interactions and in the resistance to the host defense reactions. Besides, because of the specific composition of the cell wall, enzymes involved in the biosynthesis are targets for the development of new antifungal drugs. For these reasons, we investigated the structure of the cell wall of the opportunistic fungal pathogen *Aspergillus fumigatus*. Cell wall polymers are separated in two groups: alkali-soluble components are amorphous polymers whereas alkali-insoluble components are the exoskeleton of the cell wall. Using enzymatic digestions of the alkali-insoluble fraction, fractionation by liquid chromatography, several carbohydrate structures containing interpolysaccharide covalent linkages were isolated. Physico-chemical analysis showed the absence of β1-6-glucan and the presence a linear β1-3/1-4-glucan, never described in fungi. Galactomannan, chitin and β1-3-glucan were also found in this alkali-insoluble fraction. β1-3-Glucans contain 4% of β1-6 branch point, and other polymers were covalently linked to the non-reducing end of β1-3 glucan side chains. Moreover, the characterization of specific β1-3-glucanosyltransferase activities reinforces the central role of branched β1-3-glucan in the organisation of the *A. fumigatus* cell wall.

C12.25

A model for the biosynthesis of the S-layer glycoprotein of *Aneurinibacillus thermoaerophilus* DSM 10155

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The glycan part of the S-layer glycoprotein from *Aneurinibacillus thermoaerophilus* DSM 10155 is composed of identical disaccharide repeats [→4)-α-L-Rha*p*-(1→3)-D-*glycero*-β-D-*manno*-Hep*p*-(1→]$_n$. Part of the S-layer glycan biosynthesis gene cluster was sequenced. Sequence analysis allowed to identify several open reading frames responsible for biosynthesis of sugar nucleotide precursors and for assembly and export of the glycan chain.
To verify the function of proteins involved in nucleotide-activated precursor synthesis the corresponding genes were overexpressed in *E. coli* and function was assayed using HPLC methods and NMR spectroscopy. As with other prokaryotic glycoconjugates the activated form of L-rhamnose is dTDP-L-rhamnose. Different from L-*glycero*-D-*manno*-heptose, known as core constituent of lipopolysaccharides, D-*glycero*-D-*manno*-heptose is activated as GDP-D-*glycero*-α-D-*manno*-heptose. This precursor is synthetized from sedoheptulose 7-phosphate via D-*glycero*-D-*manno*-heptose 7-phosphate, D-*glycero*-α-D-*manno*-heptose 1,7-bisphosphate, and D-*glycero*-α-D-*manno*-heptose 1-phosphate, which eventually is activated by GDP.

Reference

Kneidinger B *et al* (2001) *J Biol Chem*, in press

C13. Glycochemistry/organic synthesis/enzymatic synthesis

C13.1

Synthesis of a molecular mimic of Glc_1Man_9 oligoside as potential inhibitor of calnexin binding to ΔF508 CFTR protein

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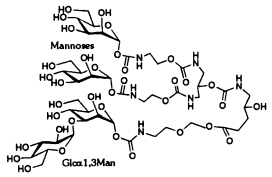
The ΔF508 mutation of the CFTR N-glycoprotein (CF transmembrane conductance regulator) involves its retention in the endoplasmic reticulum by a chaperone protein, the calnexin. The minimal structure required for a relevant chaperone binding is $Glc_1Man_5GlcNAc$. En route toward the synthesis of this oligosaccharide we already reported [1] the synthesis of disaccharide Glcα1,3Man and trisaccharide Glcα1,3Manα1,2Manα1OMe moieties. We have designed a molecular mimetic containing the determinant Glcα1,3Man and two terminal mannoses, separated by linkers. Carbamate linkages instead of glycosidic bonds were introduced to improve hydophobicity and stability. Tested for its ability to inhibit the binding of natural oligoside to His-tagged calnexin, this mimetic is a less potent inhibitor than the corresponding linear Glc_1Man_3 tetrasaccharide.



Reference

[1] Cherif S *et al* (1998) *J Carbohydr Chem* **17**:1203-1218

C13.2

Enzymatic synthesis and biochemical characterization of nucleotide-activated di- and trisaccharides

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We report on the optimized syntheses of nucleotide-activated di- and trisaccharides using nucleotide monosaccharides as acceptor substrates for glycosidases. The structural characterization of the isolated products by MS, 1D and 2D NMR revealed regiospecific transglycosylation reactions for all glycosidases. The products were tested as substrates or inhibitors of glycosyltransferases, e.g. GnT I and GnT II, β4Gal-T1, α3Gal-T, core 2 β6GlcNAc-T, and sucrose synthase. The transfer of a disaccharide unit from a nucleotide disaccharide could not be observed. In contrast, the key enzyme in O-glycan biosynthesis, core 2 β6GlcNAc-T, was inhibited by Gal(β1-4)GlcNAc(1α-UDP **1** (UDP-LacNAc), a compound in human milk [1], Gal(β1-4)Gal(β1-4)GlcNAc(α1-UDP **2**, and UDP-LacDiNAc **3**. Kinetic analyses revealed a competitive inhibition of **1**, **2**, and **3** to the donor UDP-GlcNAc (K_m 1.35 mM) with K_i values of 0.9 mM, 2.2 mM, and 3.6 mM, respectively . We conclude from our data that **1** may serve *in vivo* as a regulator of core 2 β6GlcNAc-T activity.

Reference

[1] Kobata A (1963) *J Biochem* **53**:167-175.

C13.3

Synthesis of various galactosyl donors for the glycosylation of chitooligomers

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Chitooligomers and their derivatives show unique bioactivity, e.g. they display immunostimulating effects such as acceleration of wound healing, regeneration of bone tissue and inhibition of tumor growth.
Activation of natural killer cells (NK), which are part of the immune system and responsible for recognition of virus-infected and tumor cells, takes place when specific carbohydrate ligands bind to the killer cells` transmembrane receptor proteins. In order to investigate which ligands activate the NK cells, various carbohydrates were tested with respect to their binding affinities to the receptor protein NKR-P1 [1]. It has been shown that chitooligomers have a high affinity, which increases if the terminal GlcNAc moiety is displaced by a GalNAc residue [2].
Therefore, it was of interest to synthesize and test chitooligomers with a modified terminal galactosyl residue. Glycosylation between the derivatised galactosyl donor and the chitooligomer acceptor was be carried out by chemo-enzymatic methods employing β-galactosidase from *Bacillus circulans* as catalyst. In this work, the synthesis of different galactosyl donors and their ability to function as a substrate for β-galactosidase is presented.

References

[1] Bezouska K *et al* (1994) *J Biol Chem* **269:**16945-16952
[2] Kren V *et al* (1998) *Carbohydr Res* **305:**517-523

C13.4

Synthesis of the fluorescent 2,3-alkanoylglucosamine derivatives

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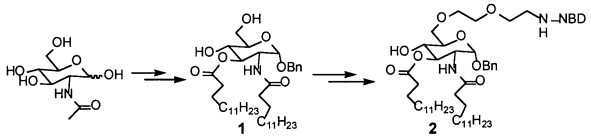
Lipopolysaccharide (LPS) is the major constituent in gram-negative bacteria and effects diverse endotoxicities. In an effort to provide an efficient probe for the mechanical study of the LPS-mediated actions in the host, the fluorophore attached 2,3-dialkanoylglucosamines are designed and synthesized. A model glycoconjugate **2** is prepared by the connection of 7-nitrobenzo-2-oxa-1,3-diazole (NBD) as a fluorophore, to the 6-position of the glucosamine **1**. The connection is proceeded by the substitution reaction of NBD-CH_2X (X=OMs, I) with the 6-hydroxyl group of the corresponding glucosamine. The glucosamine **1** is derived from *N*-acetyl-2-aminoglucoamine *via* usual selective protection/deprotection of the multi-functional groups.



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[2] Onishi HR *et al* (1996) *Science* **274**:980-982

C13.5

Chemoenzymatic synthesis of biotin-tagged UDP-Gal and UDP-GalNAc for the transfer by glycosyltransferases

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We report on the combination of an enzymatic and chemical reaction in one-pot which led to the efficient synthesis of UDP-6-biotinyl-Gal(NAc) in a 100 mg scale. The products were characterized by mass spectrometry (FAB, MALDI-TOF) and 1D/2D NMR spectroscopy. It could be demonstrated for the first time, that the human recombinant galactosyltransferases β3Gal-T5, β4Gal-T1, and β4Gal-T4 mediate biotinylation of the neoglycoconjugate BSA-$(GlcNAc)_{17}$ and ovalbumin by using UDP-6-biotinyl-Gal as donor substrate. The detection limits of Gal-biotin-tagged GlcNAc residues on BSA-$(GlcNAc)_{17}$ were in the range of 150 pmol in western blot analysis and 1 pmol in a microtiter plate assay. The degree of Gal-biotin transfer onto under-galactosylated hybrid N-glycans present at the single glycosylation site of ovalbumin was dependent on the Gal-T used, indicating that the acceptor specificity may direct the transfer of Gal-biotin. The potential of UDP-6-biotinyl-Gal as a novel donor substrate for human galactosyltransferases lies in the targeting of distinct acceptor structures, e.g. under-galactosylated glycoconjugates related to diseases, the detection of O-GlcNAc sites or the quality control of glycosylation of recombinant and native glycoproteins.

C13.6

Specific glycoligands; synthesis of cycloglycopeptides with potent biological activity

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Cycloglycopeptide as specific ligand of influenza virus infection was synthesised by combined use of chemical and enzymatic strategy. Hemagglutinin consisting of hetero trimers is exposed on the surface of influenza virus. The molecular recognition between hemagglutinin and sialic acids of the host-cell surface leads the cell-virus adhesion stage. As novel type of inhibitor of influenza virus infection, we designed and synthesised cycloglycopeptide carrying sialic acids pendants.
Firstly, cyclo[Gly-Ser-Gln-Ser-Ser-Gly]$_3$ and cyclo[Gly-Gln-Ser-His-Asp-Ser-Gly]$_3$ were chemically synthesised as scaffoldings. Next, lactose derivative having alkylamino group at reducing end was transferred at the glutamine residues of the cyclopeptide using transglutaminase-catalyzed reaction. Further sugar elongation reaction with sialic acid was subsequently achieved by employing α-2,3-sialyl-transferase to yield the target compound. Structural evaluation, biological activity and hydrolytic activity of these compounds will be discussed.

C13.7

New effective synthesis of normuramic analogs of muramic glycopeptides

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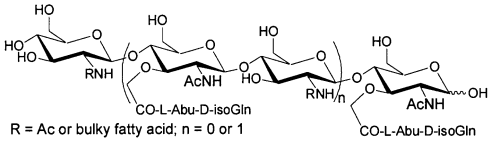
Normuramic glycopeptides in which muramic acid is replaced by normuramic acid show, in comparison with muramic glycopeptides better, immunopharmacologic parameters and some of them seem to be promising candidates as immunotherapeutics. In a classical approach to the synthesis of their saccharide part the glucosamine unit, protected on OH(3) by allyl group, is used as a precursor for normuramic acid unit, into which it can be transformed by a two-step splitting-off of allyl group (via rearrangement of acidolabile propenyl group) and a susequent introduction of glycoloylether residue to the position C(3). Here we present a simple one-step transformation based on oxidative cleavage of C=C bond of 3-*O*-allyl group which provides desired glycoloylether residue. This procedure was successfully applied to the synthesis of the depicted nonpyrogenic normuramic glycopeptides of a high immunopotentiating activity. (This work was supported by grant GACR 203/00/0071, grant MPO PZ-Z2/25/98 and research project Z4/055/905).



Reference

Ledvina M *et al* (1998) *Collect Czech Chem Commun* **63**:577-589

C13.8

Chemical engineering of bacterial cell wall

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Muramyl peptide compounds are key compounds in bacterial cell wall biosynthesis. In this work, we synthesized three fluorescein-attached muramyl peptide derivatives to test incorporation into bacterial cell wall through the biosynthesis. *E. coli,* was grown in the presence of these muramyl peptide derivatives and the cell wall component was collected to measure fluorescence. Fluorescein-labeled UDP-muramyl peptide was found to incorporate into cell wall. The result suggests that this method is applicable to chemical cell-surface engineering.

C13.9

A highly practical synthesis of potent L-selectin blockers: significant role of 6-sulfo-GlcNAc residue

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Selectins are known to be associated with the rolling of leukocyte to endothelial cells, which lead to inflammation, reperfusion injury, rheumatoid arthritis, and metastasis. Hence, a number of studies have been focused on developing artificial selectin blockers. Our group has recently proposed an efficient synthetic way towards P- and L-selectin blokers, which is based on segmentation of sialyl Lewis X (sLe^X) into polymerizable simple sugar modules and then regeneration of sLe^X mimic on polymer chains by radical copolymerization. In the present study, we segmented the structure of 6-sulfo-sLe^X into acrylamide monomers of each 6-sulfo-GlcNAc and sLe^X and copolymerized with acrylamide. The sLe^X/6-sulfo-GlcNAc/acrylamide ter-polymer, thus derived, showed potent blocking activity for L-selectin (IC_{50}= 2.8 μM) more than for P- (6.0 μM) and E-selectins (>100 μM). A copolymer without 6-sulfo-GlcNAc showed less activity for L-selectin (6.6 μM). To our surprise, a copolymer without sLe^X showed high activity for L-selectin (2.6 μM) indicating its major role in the binding to L-selectin.

Reference

Nishida Y *et al* (2000) *Biomacromolecules* **1**:68-74

C13.10

Synthetic studies on antifreeze glycoproteins (AFGPs): structure-function relationships in antifreeze glycoproteins

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Structure-function relationships of antifreeze glycoproteins [AFGPs: {Ala-Thr(Galβ1-3GalNAcα)-Ala}$_n$, n=4-50] were studied thorough chemical syntheses and activity evaluations of AFGP (**1**) and its analogues (**2-8**). AFGPs in the serum of polar fishes have been known to depress the freezing point of their blood and help them to survive at temperature below –1.9 °C. In this study, AFGP (**1**) and its analogues (**2-8**) were successfully synthesized *via* simple polymerization of their repeating units using DPPA method. Artificially prepared AFGP (**1**) showed similar activity as that of wild-type AFGP. On the contrary, neither AFGP repeating unit glycopeptide nor sugar-lacked polypeptide (**8**) did not show the activity. Surprisingly, analogues in which sugar moieties were varied to GalNAc (**2**) and LacNAc (**3**) showed similar activity as (**1**), while Galactose (**4**), Lactose (**5**), β-O-linked (**6**), and ASA-glycoprotein (**7**) did not. These results clearly indicate the importance of (i) sugar moiety, (ii) repeating structure, (iii) 2-acetamide group, (iv) α-threonyl linkage between sugar and peptide, for the specific interaction with ice. CD spectra of active compounds (**1-3**) were obviously different from inactive analogues (**4-8**), suggesting the presence of an ordered structure different from α-helix and random coil.

C13.11

Combinatorial synthesis of oligosaccharides in solution-phase and on solid-phase

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Carbohydrates and glycoconjugates play key roles in crucial biological processes. An oligosaccharide library may be an efficient tool of the research of these processes. We wish to report the syntheses of oligosaccharide library in solution-phase and on solid phase. In solution phase, we developed one-pot two- to six-step glycosylation in order to synthesize di-, tri-, tetra-, penta- hexa-, and heptasaccharides. The one-pot glycosylation is a simple method for sequential glycosyl-bond formation carried out in the same flask. We demonstrate library syntheses of heptasaccharides having phytoalexin elicitor activity for soybean. For solid-phase synthesis, a phenylsulfonyl linker was developed. The linker is stable under acidic conditions for a glycosylation with a thioglycoside or a glycosyl fluoride, and can be easily cleaved by nucleophlic addition such as an azide, an iodide, and a hydride. A combinatorial synthesis of a trisaccharide library is demonstrated by using monosaccharide cores immobilized by the linker. The utilization of manual and automatic synthesizer in the synthesis is also present.

Reference

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C13.12

Construction of oligosaccharide library by cells (13). Glycosylation of *N*-acetylglucosamine-bearing saccharide primer

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There are several methods to obtain useful oligosaccharides, involving extraction from nature, chemical synthesis, and enzymatic synthesis. We have been developing a new methodology using living animal cells to construct oligosaccharide library. In this method, analogs of glycosphingolipids, so called "saccharide primer", were administered to cells and they are glycosylated in biosynthetic pathway of cells.
HL60 cells were co-cultured with *N*-acetylglucosamine-bearing saccharide primers in serum-free medium. HL60 cells are known to express the biosynthetic pathway of neolacto series. The glycosylated products were extracted from medium fraction and cell fraction, and were analyzed by HPTLC, MALDI-TOF MS and enzyme digestion. Interestingly, the glycosylated products were almost secreted to the medium. Structures of glycosylated products were estimated from the biosynthetic pathway of HL60 cells and the result of MALDI-TOF MS, and were suggested to be the oligosaccharide sequences of neolacto series such as Lex and sLex. In conclusion, *N*-acetylglucosamine-bearing saccharide primer was a useful primer to construct an oligosaccharide library.

C13.13

Regioselective *N*-acylation of Amikacin using *p*-nitrophenyl benzoates; A simple approach towards oligomerization and chemical manipulations of aminoglycoside antibiotics

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The appearance of methicilin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) is alarming against the stream of modern therapy depending on new and newer antibiotics. Recently, many interests have been directed to carbohydrate cluster effect increasing the adhesion to proteins. An anloguous effect is expected for amino glycoside anitibitics in the interaction with ribosomal RNAs. In this study, we investigated a general way to convert *Amikacin* to variable types of multivalency models *via* regioselective *N*-acylation as the key step. The amino glycoside antibiotic has four amino groups (NH_2-*1*~NH_2-*4*), possibly showing different reactivity with acylating agents from each other. Use of *p*-vinyl-benzoyl chloride under Shotten-Baumann conditions showed regioselectivlty at NH_2-*1* (75%) and NH_2-*2* (25%). The selectivity was further increased by using *p*-nitrophenyl *p*-vinylbenzoate, NH_2-*1* (98%) and NH_2-*2* (2%). Use of the other *p*-nitrophenyl benzoates showed similarly high selectivity. This finding enabled us to prepare a series of Amikacin-based multivalency models, including an Amikacin polymer and an acrylamide/Amikacin copolymer.

C13.14

Synthesis of betaglycan tetraosyl hexapeptide: A possible precursor for heparin biosynthesis

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The sorting mechanism of the glycosaminoglycans (GAG) is of great interest in biochemistry and bioindustry. The enzymatic transfer of the first hexosamine residue: α-GlcNAc or β-GalNAc to the first GlcA determines the category of the GAG into heparin- or chondroitin-type, respectively. The exact mechanisms of the diversification of the GAG are still obscure. However, isolated heparin and heparan sulfates possess acidic and hydrophobic consensus peptides close to the glycosylation site. In addition, some artificial hydrophobic primers, such as MU xylosides can elongate to heparin or heparan sulfates. The significant signal might reside close to the core-peptide controlling the activity of the first *N*-acetylhexosaminyltransferases. These facts prompted us to elucidate the biological mechanism from the point of the synthetic chemistry. We chose a part of betaglycan, GlcA-Gal-Gal-Xyl-SGW-PDG, having acidic and hydrophobic amino acids. This proteoglycan is a possible precursor of heparin biosynthesis. Stepwise addition to the xylosyl acceptor allowed us to obtain the tetraosyl imidate which was coupled with SerGly. Final coupling of the tetraosyl serylglycine and tetrapeptide afforded the targeted tetraosyl hexapeptide.

Reference

Esko JD and Zhang L (1996) *Curr Opin Struct Biol* **6**:663-670

C13.15

Membrane-permeant derivatives of mannose-1-phosphate

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The carbohydrate-deficient glycoprotein (CDG) syndrome type I is a genetic disorder in the endoplasmic reticulum (ER) [1]. Lack of phosphomannomutase results in an effective disturbance of the biosynthesis of glycoproteins. In order to overcome the problems arising from this *in vivo* glycosylation disfunction, membrane-permeant derivatives of Man-1-P were required and synthesized. By employing the phosphoamidite [2] and the H-phosphonate methods [3] in common with biologically reversible phosphate protective groups [4] appropriate phosphotriesters and phosphodiesters could be obtained.

References

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C13.16

Design and chemo-enzymatic synthesis of ligands selective for P-selectin

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P- and E-selectin are involved in the development of inflammation related diseases like atherosclerosis by mediating the process of adhesion and rolling of leukocytes across the inflamed endothelium. In particular, P-selectin was shown to be selective and specifically upregulated in atherosclerotic tissue. As such, antagonists of P-selectin are therefore of great therapeutic value to intervene in atherosclerosis. As the natural ligand of P-selectin, PSGL-1, is synthetically eleborate and also displays affinity for E-selectin, we set out to device specific and potent PSGL-1 mimics by chemo-enzymatic synthesis. To enhance the specificity towards P-selectin, we took advantage of the fact that there are minor differences in binding requirements to both receptors. Hence, starting from glucosamine a compound library of glucosyl aldehydes was synthesized via a seven step pathway, which were then condensed to dihydroxy-acetone phosphate under the agency of fructose diphosphate aldolase. To gain enhanced interaction to the binding pocket several aromatic and aliphatic side chains were introduced. Preliminary binding studies show enhanced affinity of the lead compounds over sLeX.

C13.17

Synthesis of fragments of the glycocalyx glycan of the parasite *Schistosoma mansoni*

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Human schistosomiasis is one of the major parasitic diseases, second only to malaria in generating morbidity and suffering in tropical zones word-wide. During the cercarial stage of the life cycle of *Schistosoma mansoni*, the entire surface of the parasite is covered by a 1 μm thick, highly immunogenic, glycocalyx, containing O-linked fucosylated carbohydrates. Here, the chemical synthesis of

α-L-Fuc*p*-(1→3)-β-D-Gal*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*-(1→O$(CH_2)_5NH_2$

β-D-Gal*p*NAc-(1→4)-[α-L-Fuc*p*-(1→3)-]β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*-(1→O$(CH_2)_5NH_2$

α-L-Fuc*p*-(1→3)-β-D-Gal*p*NAc-(1→4)-[α-L-Fuc*p*-(1→3)-]β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*-(1→ O$(CH_2)_5NH_2$

is shown. These structures represent terminal sequences of the glycocalyx glycan, and in protein-conjugated form they are potential diagnostics in searching for antibodies, raised against the glycan, in serum of infected humans.

C13.18

Sialopeptides: An investigation of their mimicry of Siglec binding oligosaccharides

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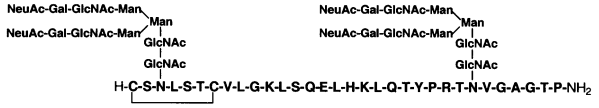
Complex oligosaccharides bearing terminal sialic acid residues are involved in a variety of biological phenomena, such as cell differentiation, inflammation, or tumor progression and metastasis. To facilitate biological studies of these processes, functional mimics of complex, sialic acid containing carbohydrates are required. Glycopeptides have proven to be highly efficient mimics of carbohydrate ligands in receptor binding assays. The goal of the research project was to identify sialic acid containing glycopeptides (sialopeptides) that bind with high affinity to sialic acid recognizing receptors like Siglec-1 using combinatorial library approaches.
The research project focuses on various aspects of this combinatorial chemistry approach including the synthesis of different sialic acid containing building blocks and their application in the synthesis of sialopeptide libraries. The screening of the libraries with Siglec-1 resulted in the identification of several sialopeptides, using MALDI-TOF MS, with high sequence consensus. The interaction of Siglec-1 with the identified sialopeptide ligands was examined and quantified using a variety of binding assays.

C13.19

Synthesis of calcitonins glycosylated at two sites: The three-dimensional structure and biological activity

K Haneda[1], T Inazu[1], M Takeuchi[1], K Toma[1], M Tagashira[2], H Iijima[2], K Kobayashi[3], M Takeuchi[3], K Yamamoto[4] and K Takegawa[5]
[1]The Noguchi Institute, Tokyo; [2]Asahi Kasei Co., Shizuoka; [3]Kirin Brewery Co., Kanagawa; [4]Graduate School of Biostudies, Kyoto University, Kyoto; [5]Kagawa University, Kagawa, Japan

In order to reveal the functions of oligosaccharides in glycoconjugates, calcitonin (CT) derivatives containing two N-linked oligosaccharides were chemo-enzymatically synthesized. CTs containing *N*-acetyl-D-glucosamine (GlcNAc) attached to the Asn or Gln residues at two sites of CT were chemically synthesized. Natural N-linked oligosaccharides were transglycosylated to them by endo-β-*N*-acetylglucosaminidase of *Mucor hiemalis* (Endo-M), and glycosylated CT derivatives containing two sialo complex-type oligosaccharides could be prepared. Using Endo-M and Endo-A (derived from *Arthrobacter protophormiae*) enzymes sequentially, CT derivatives containing each one complex-type and one high-mannose type oligosaccharides were also prepared. The three-dimensional structure of CTs were scarcely affected by oligosaccharides. The biological activity was not affected so much by oligosaccharides, and CT derivatives containing two oligosaccharides showed considerable biological (hypocalcemic) activity.



C13.20

Synthesis of peptide- and glycoconjugate-arrays on continuous surfaces as potential chitinase inhibitors

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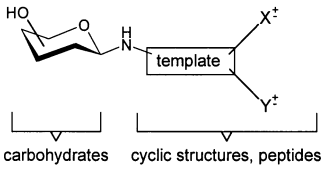
Jerini AG, Rudower Chaussee 29, 12489 Berlin, Germany

Chitinase inhibitors are of great interest as potential agents for plant protection as well as for treatment of fungal infections in human. Therefore they are highly attractive targets for development of pharmaceuticals and agrochemicals. To date only few chitinase inhibitors are known which are either isolated as natural products or prepared by traditional multistep organic synthesis [1]. To gain more information on the mode of action of chitinase inhibitors we have synthesized a large series of new compounds by solid-phase chemistry. Peptide- and glycopeptide-based structures were prepared in a highly efficient way either by multiparallel resin- or SPOT-synthesis and used for screening against several chitinases.



Reference

[1] Rottmann A *et al* (1999) *Eur J Org Chem* **48**:2293-2297

C13.21

Synthesis and characterization of glycoconjugates based on yeast cell-wall mannans

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The problem of preparation of immunogens or glycoconjugate therapeutics lies in the selection of effective conjugation methods. The chemistry of neutral polysaccharides, such as cell-surface α-D-mannans, is limited to the use of hydroxyl groups. We examined application of different strategies. The classical approach is the controlled periodate oxidation of polysaccharide that creates carbonyl groups. These groups are subsequently used for binding the spacer, adipic acid dihydrazide (ADH). Products of these reactions were examined by a combination of the following analytical methods: Park-Johnson carbonyl group assay, trinitrobenzene sulfonic acid assay of free hydrazide, elemental analysis and size- exclusion chromatography. Relatively high degree of cross-linking, even at a high excess of ADH, was found. This is a serious drawback of the strategy that involves a homobifunctional ADH linker. As a more promising method appears to be the effective activation of hydroxyl groups followed by binding to the nucleophilic groups on a protein or indirect binding through a heterobifunctional spacer. The possibility of using 1-cyano-4-dimethyl-aminopyridinium tetrafluoroborate (CDAP) for activation of mannan hydroxyl groups, instead of commonly used cyanogen bromide, was explored here. The CDAP method reveals to be simple and effective, moreover there is no danger of destruction or alteration of the polysaccharide structural features that are essential for its biological activity.

C13.22

Chemo-enzymatic synthesis of complex-type sialoglycoantigens including phospholipid groups

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Monoclonal antibodies against the sugar chains of glycoproteins are expected to be used for the determination of specific oligosaccharides occurring in liver carcinoma and cirrhosis cells. However, when we use the glycoprotein as an antigen, it is difficult to obtain the antibody because of strong antigenicity of the protein moiety in the glycoprotein. Here, we describe a chemo-enzymatic synthesis of sialoglycoantigens having a phospholipid instead of a protein moiety using the transglycosylation activity of Endo-M (endo-β-*N*-acetylglucosaminidase from *Mucor hiemalis*) and a chemical modification method.
The transglycosylation reaction between the sialoglycopeptide from hen egg yolks and the chemically synthesized glycoacceptors having few aldehyde groups was performed using a cloned Endo-M. The enzymatic reactants were purified by HPLC and analyzed by MALDI-TOF-MS and ^{1}H-NMR. Complex-type sialo-oligosaccharides having aldehyde groups were condensed with phosphatidyl ethanolamine dimyristoyl by Schiff-base reduction, leading to sialoglycoantigens.

References

Yamamoto K *et al* (1994) *Biochem Biophys Res Commun* **203**:244-252
Inoki Y *et al* (2000) *Biochem Biophys Res Commun* **276**:1210-1216

C13.23

Synthetic studies on glycosphingolipids from *Protostomia phyla*: Synthesis of arthro-series glycosphingolipids

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We have been interested in the relationships between the structure and the biological functions of glycolipids from invertebrate animal species and have so far synthesized oligosaccharides from various protostomia phyla. Recently, novel glycosphingolipids were isolated from *Lucilla caesar*, *Carabus maiyasanus* in arthropoda (Fig. 1). In the present study, we describe the synthesis of glycosphingolipids containing β-D-mannopyranosidic linkage from them. The key reaction was β-mannosylation using a highly stereoselective β-(1→4)-glycosidic bond formation by intramolecular aglycon delivery which was reported by Ito. The glycosylation of 2-deoxy-2-phthalimido-glycosyl donors with β-mannosyl disaccharide acceptor in the presence of TMSOTf gave the tri- and tetrasaccharide derivatives. The synthesized oligosaccharides were converted into the glycosphingolipid by the introduction of ceramide and then afforded the desired products by deprotection.

GlcNAcβ(1→3)Manβ(1→4)Glcβ(1)Cer
GalNAcβ(1→4)GlcNAcβ(1→3)Manβ(1→4)Glcβ(1)Cer

Fig. 1

C13.24

Synthetic galectin inhibitors

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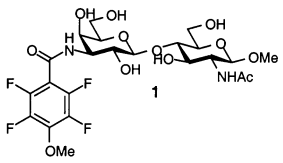
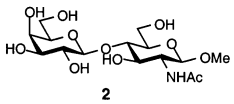
Galectins, a family of β-galactoside-binding proteins, cause a variety of cellular responses, and have been implicated in inflammation and cancer. As tools to further examine the biological functions and biomedical use of galectins we are developing synthetic high affinity inhibitors. Crystallographic data of galectin-ligand complexes reveal a highly conserved lactose/*N*-acetyllactosamine binding site as well as an extended binding site close to the 3-OH of the galactose residue. Therefore we devised strategies to derivatize this position by a variety of substituents. The derivative **1** inhibited (IC_{50} 4.8 μM) galectin-3 more than 40 times better than the parent compound **2**. X-ray crystallography of the galectin-3:**1** complex revealed a ligand induced movement (2.6A) of the Arg-144 side chain to produce a stacking interaction with the fluorinated benzamide.



1



2

C13.25

Synthesis of a new dimeric β-glycosidated muramyl peptide

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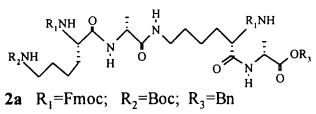
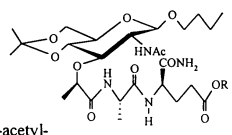
It was shown that dimerisation of MDP analogs has caused an increase of stimulatory activities against macrophage-like cells [1]. In order to evaluate the effect of dimerisation we have synthesized a dimer of the earlier obtained and tested β-butyl glycoside of MDP [2]. Protected glycopeptide **1** [2] and tetrapeptide **2a** with alternation of asymmetric centres configuration were synthesized using standard methods. These building blocks after partial deprotection were coupled by HOSU-DCC method. The final deprotection of resulting glycopeptide **2b** by treatment with trifluoroacetic acid turned out to be a particular problem owing to lactonization of the target compound **2c**. Glycopeptide **2c** was prepared after hydrolysis of obtained lactone with aqueous K_2CO_3 followed by gel filtration on Sephadex G-10.



2a R_1=Fmoc; R_2=Boc; R_3=Bn



1

2b R_1= 1-O-butyl-4,6-O-isopropylidene-β-N-acetyl-muramyl-L-alanyl-D-isoglutaminyl; R_2=Boc; R_3=Bn

2c R_1= 1-O-butyl-β-N-acetylmuramyl-L-alanyl-D-isoglutaminyl; R_2=R_3=H

References

[1] Murata J *et al* (1997) *Carbohydr Res* **297:**127-133
[2] Pertel SS *et al* (1999) *Bioorgan Khim* **25**:628-634

C13.26

The synthesis of heptasaccharides with phytoalexin-elicitor activity by one-pot multi-step glycosylation

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Carbohydrates play pivotal roles in biological processes. The structual charactarized oligosaccharides are important for the study of these processes. For the rapid assembly of the oligosaccharides we have already investigated one-pot glycosylation, which sequentially forms two and more glycosyl bonds in a vessel. The 72 member trisaccahrides were synthesized by the method using a manual synthesizer. Herein we describe the development of one-pot six-step glycosylation to synthesize heptasaccharides from seven building blocks of monosaccharide.

We established a branched-linear one-pot glycosylation using three kinds of leaving groups in order to couple four building blocks. We also found that only two leaving groups were required in the one-pot three-step glycosylation using three glycosyl donors in order to form three glycosyl bonds.

A β-(1,6) pentaglucoside having two β-(1,3) branched glucoses is a well known phytoalexin elicitor for soybean. The advanced one-pot glycosylation was applied to the synthesis of the heptasaccharide. Sequential coupling of seven building blocks was accomplished in one-pot to afford the heptasaccharide in 24% overall yield.

C13.27

Oligosaccharides to probe binding of the O-antigen of *Shigella flexneri* 2a antibodies: synthesis and binding studies

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[1]*Unité de Chimie Organique, Institut Pasteur, Paris, France;* [2]*Unité de Pathogénie Microbienne Moléculaire, Institut Pasteur, Paris, France*

Shigella flexneri 2a is a Gram negative enterobacterium that is responsible for the endemic form of Shigellosis or bacillary dysentery. As for several other pathogens, its O-specific polysaccharide (O-SP), which features the branched pentasaccharide **I** as its repeating unit [1], is a protective antigen.
As part of a program directed towards the development of chemically defined synthetic vaccines against shigellosis, we report the synthesis of the pentasaccharides **ECDAB-OMe** and **AB(E)CD-OMe** and the binding studies of a panel of synthetic mono-, di-, tri-, tetra- and pentasaccharides representative of *S. flexneri* 2a O-SP with five protective mIgGs of different isotypes, specific for this bacterial polysaccharide.

A **B** **C** **D**
2)-αL-Rha*p*-(1→2)-αL-Rha*p*-(1→3)-αL-Rha*p*-(1→3)-βD-GlcNAc*p*-(1→
(1↑4)
I αD-Glc*p* **E**

Reference

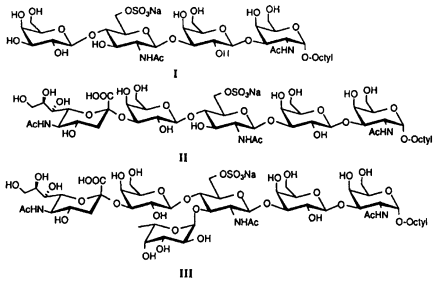
[1] Lindberg AA *et al* (1991) *Rev Infect Dis* **13:**S279-S284

C13.28

Synthesis of sulfated O-linked oligosaccharides: Epitopes for MECA-79

D Rabuka, F Belot, J-C Yeh, N Hiraoka, M Fukuda and O Hindsgaul
Glycobiology Program, Cancer Research Center, The Burnham Institute, 10910 North Torrey Pines Road, La Jolla, CA 92037, USA

Oligosaccharides present on L-selectin counter receptors have been implicated in lymphocyte homing and recirculation. The functionality of these counter receptors is dependent on their substitution via sialylation, fucosylation, and sulfation. MECA-79 antibody specifically decorates the luminal surface of high endothelial venules in secondary lymphoid organs.



Here we present the chemical and enzymatic synthesis of novel sulfated *O*-linked oligosaccharides **I-III** recognized by MECA-79 of which **I** was found to be the minimum required epitope [1].

Reference

[1] Yeh J-C *et al*, manuscript submitted

C13.29

Production of *N*-acetylneuraminic acid by bacterial coupling

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Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan

N-acetylneuraminic acid (NeuAc) at the terminal positions of glycoproteins and glycolipids plays important roles in biochemical recognition processes. Derivatives of NeuAc have been developed as drugs and diagnostics for influenza. NeuAc is still expensive, although an enzymatic synthesis of NeuAc from *N*-acetylglucosamine (GlcNAc) and pyruvate was reported using GlcNAc 2-epimerase of porcine kidney and NeuAc aldolase [1]. GlcNAc 2-epimerase catalyzes the interconversion between GlcNAc and *N*-acetylmannosamine that is a precursor of NeuAc. We cloned the gene for GlcNAc 2-epimerase from *Synechocystis* sp. PCC6803 through the homology search, and constructed *Escherichia coli* overexpressing GlcNAc 2-epimerase. We also constructed *E. coli* overexpressing NeuAc synthetase of *E. coli* that catalyzes the formation of NeuAc from ManNAc and phosphoenolpyruvate (PEP). *E. coli* cells possess the activity to form PEP from glucose through glycolysis. When these *E. coli* cells were used as enzyme sources, NeuAc accumulated from GlcNAc and glucose. In order to increase the supply of PEP, putting another microorganism, *Corynebacterium ammoniagenes*, into the reaction mixture led to higher accumulation of NeuAc. The production system using bacterial coupling can be applied to a large-scale manufacture of NeuAc.

Reference

[1] Maru I *et al* (1998) *Carbohydr Res* **306:**575-578

C13.30

Synthesis of glycopeptide analogs of Contulakin-G, a Cone snail neurotoxin

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[1]*Department of Chemistry, Swedish University of Agricultural Sciences, PO Box 7015, S-750 07 Uppsala, Sweden;* [2]*The Salk Institute, PO Box 85800, San Diego CA 92186, USA*

Cone snails are marine organisms that prey on fish by harpooning and immobilizing them with peptide-containing venoms. Several venoms from the Cone snail C. geographicus have been characterized [1], one of them is Contulakin-G, a glycopeptide with significant pain-relieving properties. It was shown, that the biological activity of this glycopeptide is significantly reduced on removal of the sugar. We have further investigated this phenomenon by synthesizing analogs of Contulakin-G with different sugar configurations.

β-D-Galp-(1→3)-α-D-GalNAcp-(1-O
|
pGlu-Ser-Glu-Glu-Gly-Ser-Asn-Ala-Thr-Lys-Lys-Pro-Tyr-Ile-Leu-OH

Contulakin-G

Synthetic methods and some preliminary biological results will be presented.

Reference

[1] Craig AG *et al* (1999) *J Biol Chem* **274**:13752

C13.31

Synthesis and characterization of glycoconjugates of poly(styrene-co-maleic anhydride)

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Poly(styrene co-maleic anhydride) (SMA) was derivatized with 1-amino-1-deoxy-β-D-galactose, 1-amino-1-deoxy-β-D-glucose and 1-amino-1-deoxy-β-lactose to afford glycoconjugate derivatives of a biocompatible polymer. The amino sugars were chemically conjugated with poly(styrene co-maleic anhydride) via formation of an amide bond between the anomeric amino group of the sugar residue and the carboxyl anhydride of poly(styrene co-maleic anhydride). Colorimetric assay of the unreacted amino groups and elemental analysis were used to determine the degree of substitution. About 56%, 54% and 94% of the available anhydride groups reacted to give galactose-, glucose- and lactose-amide branched polymer, respectively. For the glucose-amide branched polymer the degree of substitution was determined also by capillary electrophoresis (CE). By using CE, the amount of glucosamine that was released with time by the polymer derivative upon basic hydrolysis (NaOH 3 M, 60°C) was detected. In satisfactory agreement with elemental analysis and UV data, the presence of one sugar residue every two SMA repeating units was deduced from the analysis of the kinetics of hydrolysis. The synthesized glycopolymers were characterized by Fourier Transform Infrared spectroscopy (FT-IR), gel permeation chromatography (GPC) and circular dichroism (CD) spectroscopy. Interestingly, the polymer derivatives developed a rather complex CD spectra, likely, as a result of a specific mutual orientation of styrene and carboxylate chromophors, laterally positioned to the amide linked sugar.

C13.32

Synthesis of partially protected pentaerythritol

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The structural features of pentaerythritol make it suitable as a starting material for further functionalization to give partially protected analogues suitable for the synthesis of acyclonucleosides, glycosides and dendrimers. Thus, depending on the reaction condition and the type of aldehyde, two of the hydroxyl groups of pentaerythritol could be protected by the reaction with benzaldehyde or its derivatives to give a 5,5-dihydroxymethyl-dioxane and/or the respective bisdioxan with spiral junction containing no hydroxymethyl groups. However, both of the dioxan rings in the latter could be opened with *N*-bromosuccinimide to give di-*O*-benzoyl-dibromodideoxy-di-*O*-(*p*-toluenesulphonyl)-pentaerythritol. The hydroxylmethyl groups of the monodioxan analogue could be partially or fully protected depending on the reaction condition which upon opening of the dioxan ring gave derivatives with good leaving groups suitable for the synthesis of acyclonucleosides.

C13.33

Enzymatic synthesis of mucin-type O-glycans on a naturally occurring aglycon

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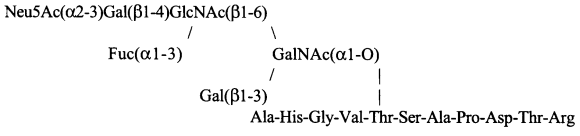
The aberrant glycosylation expressed by MUC1 in malignant tumors may be exploited for the production of glycopeptide mimics. Such mimics could be employed to prevent the spreading of cancer or for vaccination purposes. Starting from a synthetic MUC1-derived peptide (MUC1a') and using a completely enzymatic approach, the core-2 type sialyl Lewis x structure was synthesised. First, polypeptide GalNAc-transferase 3 was used to site-specifically glycosylate MUC1a' to give MUC1a'-GalNAc. Then, in a one-pot reaction employing β-galactosidase and core-2 β-1,6-*N*-acetylglucosaminyltransferase the core-2 O-glycan structure was prepared. The core-2 structure was then sequentially galactosylated, sialylated, and fucosylated by making use of β-1,4-galactosyltransferase 1, α-2,3-sialyltransferase 3, and α-1,3-fucosyltransferase 3, respectively, resulting in the sialyl Lewis x glycopeptide. The overall yield of the final compound was 23%. All products were characterized by mass spectrometry and NMR spectroscopy.



C13.34

Applications of immobilized glycosyltransferases in glycoconjugate synthesis

S Nishiguchi[1], H Nakagawa[1], S Shibatani[1], A Toda[1], M Kurogochi[3], K Yamada[2] and S-I Nishimura[3]
[1]*Laboratory for Glycocluster Project, Japan Bioindustry Association, Sapporo, 060-0810 Japan;* [2]*New Energy and Industrial Technology Development Organization, Sapporo, 060-0810 Japan;* [3]*Division of Biological Science, Graduate School of Science, Hokkaido University, Sapporo, 060-0810 Japan*

We have proposed the strategy for enzymatic synthesis of oligosaccharide derivatives on water-soluble polymer supports having unique linkers that can be cleaved by specific condition. In this paper, an application of immobilized glycosyltransferases for this strategy will be described. Some of the glycosyltransferases were prepared as fusion protein with maltose binding protein (MBP) expressed in *E. coli*. Glycosyltransferases were immobilized covalently by conventional methods using activated Sepharose. MBP-glycosyltransferase fusion proteins were also immobilized on the polymer support having maltooligosaccharide residue with affinity of MBP. These immobilized enzymes could elongate sugar chain efficiently and bear sialyllactosamine and sialyl Lewix x on the polymer support.

References

Yamada K *et al* (1998) *Carbohydr Res* **305**:443-461
Nishimura S-I and Yamada K (1997) *J Am Chem Soc* **119**:10555-10556

C13.35

Synthesis of spacered Gal(β1-4)GlcNAc(β1-2)Man mimics for exploring the substrate specificity of sialyltransferases

JAF Joosten, B. Evers, RP van Summeren, JP Kamerling and JFG Vliegenthart
Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University, Utrecht, The Netherlands

Sialic acids occur at the non-reducing termini of many glycoconjugates, and are considered to be 'key' determinants in the regulation of a variety of biological processes. In the biosynthesis of sialylated glycans a sialyltransferase family of over 10 members with different substrate specificities is involved. Currently, we have studied sialyltransferases (ST) involved in the α-2,3- and α-2,6-sialylation of terminal Gal units in N-glycoprotein glycans. The trisaccharide β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→O)$(CH_2)_7CH_3$ and eleven analogues containing structural variants of D-Gal were synthesized and employed as substrates for ST6Gal I and ST3Gal III [1]. Hydroxyl groups at either C3 or C4 of the D-Gal residue were substituted by hydrogen or fluorine, by amino- or *O*-methyl groups, or were inverted, to determine their involvement in binding and catalytic activity. In addition, trisaccharides containing α-L-Alt (inverted hydroxymethyl group at C5) or β-L-Gal (enantiomer) at the non-reducing terminus were constructed as probes. The ST6Gal I tolerated most of the modifications at the Gal residue to some extent, whereas the ST3Gal III displayed a narrower specificity [1]. In a further study we have synthesized a series of trisaccharides with modifications at C2 or C6 of the Gal residue, together with their *N*-propionylated glucosamine analogues. For the trisaccharide probes containing an *N*-acylated glucosamine ánd an *N*-acylated galactosamine unit, use was made of a combination of *N*-phthalimido and *N*-dimethylmaleoyl protection.

Reference

[1] Van Dorst JALM *et al* (1996) *Eur J Biochem* **242:**674-681

C14. Glycoconjugates in nutrition

C14.1

Identification of glycosylated α-lactalbumin as endogenous substrate for β1,4galactosyltransferase (GalT) in colostrum

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We have recently shown that spontaneous galactosylation of specific agalacto-glycoproteins occurs in colostrum due to the presence of UDP-Gal and Mn^{2+} in sufficient amounts for the catalysis of GalT [1]. In order to identify the glycoproteins susceptible to colostral GalT, dialyzed colostrum was incubated with UDP-[^{14}C]Gal in the presence of β-galactosidase. Two ^{14}C-labeled glycoproteins were detected at 23 and 18 kDa by SDS-PAGE/autoradiography. In this paper we purified and identified the 18 kDa substrate glycoprotein (18 kDa-gp). Colostrum whey was first fractionated by ammonium sulfate precipitation. The fraction rich in the 18 kDa-gp was further separated by DEAE-Sephadex chromatography, rechromatographed and subjected to Sephacryl S-200 chromatography. The identity of the 18 kDa-gp as glycosylated α-lactalbumin (gly-α-LA) was strongly suggested by three lines of evidence: (1) The *N*-terminal amino acid sequence was identical to that of α-LA; (2) After *N*-glycanase treatment the 18 kDa band shifted to the 14 kDa position corresponding to that of non-glycosylated α-LA; (3) The 18 kDa band was reactive to RCA1. Interestingly, gly-α-LA was coeluted with GalT, while non-glycosylated α-LA was eluted slower on Sephacryl S-200 chromatography of colostrum, suggesting the association of GalT with gly-α-LA but not with α-LA in colostrum.

Reference

[1] Oubihi M *et al* (2000) *FEBS lett* **236:**165-168

C14.2

Quantitative changes in milk oligosaccharides during lactation

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Human milk contains large amounts of free oligosaccharides. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was used to quantify thirteen major milk oligosaccharides. The concentrations of the individual oligosaccharides were analyzed in milk from five donors, followed separately during six to nine months of lactation. The fucosylated oligosaccharides 2-fucosyl lactose, lacto-N-fucopentaose I and lacto-N-di-fucohexaose I showed decreasing concentrations in milk during lactation. The concentrations of lacto-di-fucotetraose, lacto-N-fuco-pentaose II and III remained constant, while the concentration of 3-fucosyl lactose (3-FL) increased during lactation. The increase of 3-FL was found for all individuals independent of secretor status. The oligosaccharides containing sialic acid α2-6 linked to galactose (6-sialyl lactose and LSTc) decreased more than tenfold during lactation. In contrast, the concentration of 3-sialyl lactose (3-SL) containing sialic acid α2-3 linked to galactose remained constant during nine months of lactation. This study shows that the composition of individual oligosaccharides in milk varies considerably during lactation. The changes in milk oligosaccharide concentration may have effects on the colonization of intestinal bacteria and indirectly on the development of the immune system of the infant.

C15. Glycoconjugates in xenotransplantation

C15.1

Galα1- terminated polyglycosylceramides in porcine tissues

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The Galα1-3Gal determinant (Galα) is the major xenoantigen in pig to human xenotransplantation species combination. This antigen determinant has been identified in glycoproteins and short chain (< 16 sugar residues) glycolipids. Polyglycosylceramides (PGC) are large water soluble glycolipids with up to about 50 sugars. These compounds have blood group antigen properties and are receptors for viruses and bacteria. To date, no data regarding the presence of Galα terminated PGC xenoantigens in pig tissues has been presented.
The aim of the present study is to isolate and structural characterise Galα terminated PGC xenoantigen in pig tissues. PGC were isolated as acetylated derivatives from intestinal mucosa and erythrocytes from a blood group A pig [1]. The PGC were fractionated on Sepharose LH20 and LH60 columns, deacetylated and the reactivity with the *Griffonia simplicifolia* (*GS*) lectin was tested on TLC-plates. No reaction was observed for PGC fractions isolated from pig erythro-cytes. However, several bands were stained with the *GS* lectin and blood group A antibody in the pig intestinal mucosa PGC region. Mass spectrometry analysis of the permethylated mucosa PGC is in progress as well as the isolation of PGC from other porcine tissues, which will be discussed. In conclusion, pig intestinal mucosa contains Galα terminating PGC xenoantigens.

Reference

[1] Miller-Podraza H (2000) *Chem Reviews* **100**:4663-4681

C15.2

A second galactosyltransferase which synthesises the major xenoepitope Galα(1,3)Gal

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The major barrier for pig to human transplantation is hyperacute rejection due to Galα(1,3)Gal (Gal) antibodies, and we have reported that by the expression of α1,2fucosyltransferase (FT) almost entirely suppresses the expression of Gal in mice, however this approach is not as successful in transgenic pigs. Using monoclonal anti-Gal antibodies residual staining was found in tissues such as thymus, liver and heart of Gal o/o mice. We have isolated cDNA clones from mice and pig that encode a second α1,3galactosyltransferase (GT2), which is homologous to the rat iGb3 synthase recently isolated. Northern blot studies show that these two glycosyltransferases are expressed in different tissues. Transfection of pig or mouse into COS or CHOP cells produces Gal as detected using the IB4 lectin, purified human anti-Gal antibodies and the monoclonal anti-Gal antibodies, but in contrast to the GT1, co-expression of GT2 and FT does not produce a significant decrease in Gal. Analysis of the genomic structure show that the GT2 coding region is encoded for by 5 exons. The human homolog is located on chromosome 1, but the results of mRNA analysis suggests that this is a non-processed pseudogene.

C16. Glycoimmunology

C16.1

The immunodeterminants of antigens for the *in vivo* synthesis of anti-carbohydrate antibodies

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Glycoconjugates, glycoproteins and polysaccharides have been used to immunize rabbits and antibodies have been isolated from the serum by affinity chromatography. Inhibition, periodate oxidation and specificity tests showed that the antibodies are anti-carbohydrate. Monosaccharides (10 types) and oligosaccharides (6 types) of the carbohydrate moieties of the antigens have been shown to be the immunodeterminant residues. Some of the antibodies have been used to determine polysaccharide gums added to processed foods to improve quality, some for identification of bacterial infections and others for detecting abnormal glycoproteins in human diseased organs. Some of the latter antibodies could be useful for development of new treatment regimes. The hormone, erythropoietin (EPO), essential for the regulation of the synthesis of red blood cells has been found to contain two different carbohydrate immunodeterminant units with structure NeuAc-α(2→3)-Gal and Gal-β(1→4)-GlcNAc for *in vivo* synthesis of two types of antibodies. An immunological method for assaying EPO may be developed and used for monitoring patients on medical treatments with the hormone or to detect the use of the hormone by athletes for enhancing performance in competitive sports.

C16.2

Macrophages recognize early apoptotic T cells through the carbohydrate chains of CD43 undergoing clustering

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Apoptotic cells are selectively recognized and phagocytosed by macrophages. Carbohydrate chains have been suggested to play a role as ligands on apoptotic cells for the recognition. However, little is known about their structure, mechanism of appearance, and molecular species of glycoconjugates involved. Previously, we have demonstrated that oxidized cells are recognized by macrophages through carbohydrate chains, particularly those containing sialylated polylactosamine, and suggested that membrane glycoproteins cluster upon cell oxidation, and resultant clusters of their carbohydrate chains provide multivalent high-affinity determinants for the recognition. Here, we show that apoptotic T cells are recognized by the same mechanism. When leukemic T cells (Jurkat cells) were treated with an apoptosis-inducing agent etoposide for 1 to 2 h, they were effectively recognized and phagocytosed by leukemic THP-1 cells differentiated into macrophages. This recognition was prevented when Jurkat cells had been treated with neuraminidase or endo-β-galactosidase, or when apoptotic Jurkat cells were treated with anti CD43 or Datura lectin. CD43 glycoproteins on the apoptotic cells were found to be clustered, and this clustering was prevented by inhibition of apoptosis. These results indicate that early apoptotic T cells are recognized by macrophages through the specific carbohydrate chains of CD43 undergoing clustering.

C16.3

N- and O-linked glycans from human glycophorin A carrying blood group H antigens

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Blood group ABH antigens are well known carbohydrate structures, which represent non-reducing terminals of oligosaccharides of glycosphingolipids and glycoproteins, which are components of erythrocytes and other cell membranes. ABH antigens play a crucial role in blood transfusion, they may undergo structural changes in the tumor cells.
Glycophorin A is the major sialoglycoprotein of human erythrocyte membrane. It contains one N-glycan and *ca* 15 O-glycans in the molecule. As an erythrocyte membrane constituent it is a potential carrier of blood group ABH antigens. Previously, we have shown the presence of A and B blood group antigens in human glycophorin. The aim of this study was identification of H antigens in glycophorin, derived from blood group O red cells.
The glycophorin was degraded by β-elimination under reducing conditions; after additional degradation performed on the N-linked glycopeptides, the pools of free, reduced O-glycans and N-glycans were isolated separately. The oligosaccharides were permethylated and analyzed by nanoflow electrospray MS/MS. The product ion spectra revealed the presence of H-determinants in sialylated O- and N-glycans. The abundance of oligosaccharides carrying in glycophorin blood group H antigens is less than few percent.

C16.4

Anti-GM1 autoantibodies detection using flow cytometry: a new method for diagnosis of peripheral nervous system diseases

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Gangliosides distribution depends upon cells, tissues and animal species. GM1 is especially expressed in nervous system and particularly at the node of Ranvier. GM1 is involved in cellular differentiation, activation and adhesion. Sugar moieties of GM1 can be also target for autoimmune response. Low titers of natural anti-GM1 antibodies could be detected in sera of healthy individuals. High affinity anti-GM1 antibodies are frequently described in several nervous system diseases, mainly in motor neuropathy with persistent conduction blocks and axonal forms of Guillain-Barré Syndrome. ELISA and Immuno-TLC do not fully account of the membrane environment which acts on antigen presentation. We developped a method for anti-GM1 autoantibody detection using flow cytometry. Exogenous GM1 was incorporated in membrane of a selected cell line and autoantibodies binding was compared with that observed on non-treated cells. The reliability of this method was established by analyzing more than 80 sera. Results, compared with those obtained with ELISA, showed that this method was more specific for the diagnosis of peripheral neuropathies .

C16.5

A new neutral glycosphingolipid of human erythrocytes responsible for the NOR polyagglutination

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The NOR polyagglutination has been described so far in two families. The NOR RBCs contain two unique neutral glycolipids detectable on thin-layer chromatography (TLC) plates with *Griffonia simplicifolia* IB4 lectin (GSL-IB4) [1]. The structure of one of them (NOR1) was studied by ion trap mass spectrometry, and by treatment with α-galactosidase and β-hexosaminidase, followed by identification of products on TLC plates by lectins and anti-Gb_4 monoclonal antibody. The results showed that NOR1 is an α-galactosylated globoside, Galα1-4GalNAcβ1-3Galα1-4Galβ1-4Glc-Cer (IV^4-α-Gal-Gb_4Cer). Although NOR1 reacted strongly with GSL-IB4, which recognizes preferably the Galα1-3Gal sequence, it did not react with human anti-Galα1-3Gal antibodies. These antibodies were purified by affinity chromatography on porcine glycophorin and reacted strongly with IV^3-α-Gal-nLc_4Cer (α-galactosylo-paragloboside) isolated from rabbit RBCs. Anti-NOR agglutinins, isolated by adsorption to glutaraldehyde-fixed NOR RBCs and elution with galactose, reacted with both NOR glycolipids, but not with the rabbit glycolipid. Therefore, anti-NOR agglutinins may represent a new type of human anti-carbohydrate antibodies with specificity for Galα1-4GalNAc.

Reference

[1] Kusnierz-Alejska G *et al* (1999) *Transfusion* **39**:32-38

C16.6

A novel mechanism of host carbohydrate recognition by the C-type lectins DC-SIGN and DC-SIGNR

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DC-SIGN and DC-SIGNR are novel cell-surface C-type lectins that mediate carbohydrate-dependent interactions between immune cells via ICAM-3 binding. Both proteins also bind to the gp120 coat protein of HIV-1, contributing to virus trafficking. These molecules share 77% sequence identity at the amino acid level and are type II trans-membrane proteins in which the extracellular domain comprises a neck made up of seven 23-residue tandem repeats and a C-terminal C-type lectin domain. Soluble recombinant fragments of DC-SIGN and DC-SIGNR were expressed and purified using mannose affinity columns. For both proteins, crosslinking and equilibrium ultracentrifugation studies showed that the CRD plus neck fragments exist as stable tetramers whereas the CRDs are monomeric. Circular dichroism spectroscopy indicated that the neck structures are alpha helical. Both DC-SIGN and DC-SIGNR show a preference for mannose-like monosaccharides. Affinity for Man-9 oligosaccharide is considerably greater, even for the monomeric CRDs. Further increased affinity was seen using a dimeric glycopeptide bearing two Man-9 sugars. These results provide strong evidence for extended oligosaccharide binding sites in the CRDs of these molecules, possibly presenting a novel means of amplifying specificity for clustered host carbohydrates.

C16.7

The high carbohydrate possessing antigen (HM1) in mite feces activates CD11b-cell and produce IFN-γ from T cell

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Sensitization to allergens derived from the house dust mites of the genus *Dermatophagoides* is closely related with symptoms and rhinitis in many parts of the world. We report a novel mite allergen with high carbohydrate content and with high molecular weight (over 70k Da), which induced the proliferation of non-immunized mouse spleen cells and human peripheral blood cells without B cells. Analysis of sandwich ELISA and Flow cytometry indicated that HM1 proliferated T cells under the co-existence of CD11b-cell and produced IFN-γ. We demonstrated that the proliferative ability did not change after pronase digestion of HM1 but completely loss after periodate oxidation. Moreover, examination in vivo suggested that HM1 antigen would produce IFN-γ and aggravate cellular inflammation. We expected that the carbohydrate region of HM1 allergen would play an important role for changing Th1/Th2 balance to Th1-type T cell.

C16.8

Absorption of anti-blood group A antibodies by a recombinant mucin

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Aim: To evaluate the anti-blood group A antibody absorption efficacy of a recombinant mucin carrying blood group A determinants, for possible use prior to incompatible ABO allotransplantation.
Methods: Recombinant mucins substituted with blood-group A determinants was produced by transfecting various host cells with cDNAs encoding a mucin/immunoglobulin (Ig) chimera, FUT1 or FUT2 and the A gene. Blood group A epitope density was evaluated by ELISA. The absorption efficacy was evaluated by hemagglutination and an anti-blood group A ELISA.
Results: The recombinant mucins expressed in CHO, COS and 293T cells all carried blood group A determinants, with the highest determinant density on mucins made in 293T cells with the A gene and FUT2. The RBC agglutination by human serum absorbed on 18 pmol of these mucins, carrying approx. 1.8 nmol of A-trisaccharide, was decreased by 2 titre steps. The level of anti-A IgA/G/M dropped by 58 %. The corresponding values for 100 mg of A-PAA-MPG (200 nmol A-trisaccharide) were 4 titre steps and 94 %, respectively. However, the non-specific absorption of serum proteins was much higher with the latter absorber compared to the mucin.
Conclusion: A recombinant mucin/Ig carrying blood group A determinants was produced and shown to be an effective absorber of anti-A antibodies.

C16.9

Sialyl-Le^X on O-glycan in human pre-B lymphoid cells is synthesized on β1->4GalT-I-dependent backbone structure

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Roles of the β1->4GalT gene family are currently under investigation. Among them, β1->4GalT-I, -IV, and -V possibly biosynthesize O-glycan structures. Meanwhile, we have revealed that sialyl-Le^X (sLe^X) antigen determinant is mainly expressed on O-glycans of a single glycoprotein (gp150) in human pre-B cells, and that down-regulation of sLe^X expression is controlled by core 2 GlcNAc-transferase-I during differentiation. In the present study, we have investigated possible roles of β1->4GalT-I, IV, and V in human pre-B cell line KM3 transfecting antisense cDNAs. After transfection, we have established several transfectants, and selected the most overexpressed sublines. Flowcytometry analysis revealed that cell surface sLe^X expression level was significantly suppressed by the antisense β1->4GalT-I transfection. In addition, the suppression was also confirmed by immunoblot and E-selectin-overexpressed COS cell adhesion assay. Although the antisense β1->4GalT-IV transfection resulted in the reduced capability of cell adhesion, the endogenous expression was very low in KM3 cells and the suppression of sLe^X expression in flowcytometry analysis was not so significant as that of antisense β1->4GalT-I transfectant. So, these results strongly suggest that sLe^X on O-glycan in human pre-B lymphoid cells is biosynthesized on β1->4GalT-I-dependent backbone structure.

C16.10

Cloning and characterization of anti-GM1 autoantibodies from a patient with Multifocal Motor Neuropathy (MMN)

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Although anti-ganglioside antibodies thought to be important in the pathogenesis of immune-mediated neuropathies, such as MMN, few antibody sequences have been obtained from these individuals. We used Fab-phage display methods to examine the repertoire of anti-GM1 antibodies in MMN. To this end, Fab-phage libraries (γ1; κ + λ) were prepared using peripheral blood lymphocytes from a patient with MMN who had high serum titers of IgG anti-GM1 and from a normal individual who lacked serum anti-ganglioside antibodies. After 4 rounds of panning against purified GM1, no clones with anti-ganglioside specificity were obtained from the normal individual. In contrast, 39 different GM1-specific clones were isolated from the MMN patient's library. Sequencing revealed that 26 of these 39 clones used variable regions from the VH3 gene family, with most of these using either the DP-31/V3-9P or DP-51+ VH genes, the D6-13/DN1 D gene, and the JH4b J segment. In contrast, multiple different Vκ and Vλ genes were used. The heavy chain was critical in determining antigen specificity and affinity; for example, although clones #1-4 and #2-29 bound equally well to GM1 and had identical heavy chain sequences, their light chains were completely different, one using Vλ 3r.9c5/DPL23 and the other using Vκ EV15+. To our knowledge, these are the first results examining the diversity or restriction of the autoantibody repertoire in MMN.

C16.11

Analysis by surface plasmon resonance of anti-carbohydrate responses to synthetic glycan epitopes in *Pan troglodytes* vaccinated with irradiated *Schistosoma mansoni* cercariae

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Schistosomiasis vaccination studies have shown that humoral anti-carbohydrate responses greatly contribute to protective immunity against this debilitating disease. A group of vaccinated and control animals were followed for the duration of 1 year after infection with *Schistosoma mansoni.* Using surface plasmon resonance spectroscopy, the presence of serum IgM and IgG antibodies to a panel of schistosome-specific synthetic glycan epitopes was monitored. Strong IgG antibody responses were mainly directed to mono- and difucosylated LacdiNAc epitopes containing Fucα1-3GalNAc or GalNAcβ1-4-(Fucα1-2Fucα1-3)GlcNAc motifs. Interestingly, these glycans are related to nonreducing terminal oligosaccharide sequences found in both infectious cercariae and the pathology related eggs. Lower antibody responses were measured to the LacdiNAc epitope and glycans related to the polysaccharide chains of the circulating antigens CAA and CCA of *S. mansoni.* These data provide new insights in the humoral immune response to schistosome-derived glycans.

C16.12

Immunochemical investigations of *Ralstonia solanacearum* lipopolysaccharides

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The serological reactions between *Ralstonia solanacearum* strains were investigated. A total 8 polyclonal O-antisera, raised against heat-killed bacterial cells and containing anti-lipopolysaccharide antibodies, were tested with the reference to lipopolysaccharides (LPS) of 8 strains which differed by O-PS structures. In the reaction of ring precipitation, agglutination, Ouchterloni double diffusion, immunoelectrophoresis and ELISA test it was shown that LPS of all *R. solanacearum* strains were serologically active with homological antiserum. The data obtained in cross reactions in Ouchterloni double diffusion, rocket immunoelectrophoresis and ELISA test indicated: (1) LPS of type *R. solanacearum* strain 5712 which is differed from LPS of strains 8110, 7945 and 7955 by configuration (α- or β-) or/and type of link (1→2 or 1→3) of GlcNAc with rhamnose residue or by the absence of a side chain in their O-PS structure interacts with antiserum raised against above mentioned strains; (2) LPS of *R. solanacearum* strains 767 and 7944, except of homological system, interact only with antiserum to type 5712 strain; (3) *R. solanacearum* strains 4157 and 8089 LPS, which O-PS structures are differed both from each other and from O-PS structures of the rest strains tested were serologically inert against the heterological antiserum. This way, *R. solanacearum* is serologically heterogeneous species, including, at least, five serogroup.

C16.13

Internalization of GD1c ganglioside on activated mouse helper T lymphocytes

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Mouse immunocytes express several unique gangliosides, which are synthesized from asialo-GM1 as a biosynthetic precursor. One of the gangliosides is GD1c (NeuGc-NeuGc-), NeuGcα2-8NeuGcα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-Cer. We reported that GD1c is restricted to a small population of murine mature thymocytes and part of helper T cells. In the present study, GD1c expression was analyzed in naive and activated T cells. All naive cells ($CD44^{low}CD45RB^{high}$) expressed GD1c before activation. After ConA or anti-CD3 mAb activation, GD1c decreased and then disappeared from the cell surface. However, GD1c was detected as patches inside the activated cells, indicating that GD1c is internalized upon the cellular activation. The presence of GD1c was also confirmed by chemical detection in lipid fraction extracted from the activated cells. In contrast to the disappearance of GD1c, cell surface expression of asialo-GM1 significantly increased upon the activation, indicating that there are at least two kinds of glycosphingolipids that behaves quite differently during the cellular activation. We are planning to analyze co-localization of these glycosphingolipids with various cell surface molecules such as CD3, CD5, CD2 and CD28.

Reference

Nakamura K *et al* (1995) *J Biol Chem* **270:**3876-3881

C16.14

Effect of peptido-glucan in Maitake mushroom on control of T lymphocytes Th-1/Th-2 proportion

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The peptidoglucan in Maitake mushroom showed the strong anti-tumor effects. We investigated on the anti-tumor functions of this peptido-glucan, named D-Fraction, in relation to its proportion control on T lymphocyte Th-1/Th-2 under tumor-immunity and on the involvement of Cytokine in it.
The results were (1) Intraperitoneal administration of D-Fraction inhibits the antibody production from B cells at tumor-located area and enhances activation of helper T cells; (2) D-Fraction invites IFN-β and TNF-α expression, enhances IL-12 p70 and IL-18 production, as well as inhibit IL-4, and in the consequence establishes Th-1 dominance in the Th-1/Th-2 proportion that was Th-2 dominant due to tumor-inhibition; (3) D-Fraction was observed to inhibit IL-4 expression by NKT cell and to inhibit at the early stage of immune reactions transmigration of Th-2 cells from naive T cell (Th-0) and to establish the dominance to Th-1 cells. These facts indicate that the process in which D-Fraction expressed anti-tumor effects is also attributed to Th1 dominance which induces the cellular immunity.

C16.15

Involvement of protein kinase C in the action of Maitake polysaccharide for NO release from RAW 264 cells

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We have reported that D-fraction extracted from maitake *(Grifola frondosa)* activates host defense systems *in vivo*, and enhances antitumor activities. In this study, we found that the direct application with D-fraction to RAW 264 cells *in vitro* induced NO release. However, the intracellular pathways that cause NO release from RAW 264 cells after the application of D-fraction are not well known. To elucidate the involvement of protein kinase C (PKC) in D-fraction-stimulated NO release, we examined the effects of various PKC inhibitors on the NO release. Staurosporine, a potent protein kinase inhibitor and calphostin C, a specific PKC inhibitor, inhibited D-fraction-stimulated NO release from RAW 264 cells, while RT5720, H-8 and Rp-cAMPS (inhibiors of cyclic AMP-dependent protein kinase) showed no effect. In addition, rottlerin, a specific inhibitor of PKCδ, inhibited the effect of D-fraction on the NO release, while Go 6976 (a specific inhibitor of PKCα and βI) did not affect the NO release. By immuno-blotting analysis, the expression of inducible type of NO synthase (iNOS) was induced by D-fraction, and the expression was inhibited by rottlerin. In addition, D-fraction was found to induce activation of PKCδ. The different effects of PKC inhibitors on the NO release suggest the involvement of PKCδ in D-fraction-stimulated NO release from RAW 264 cells.

C16.16

A preliminary study of the immune response to a synthetic *Haemophilus influenzae* type b oligosaccharide conjugate to *Neisseria meningitidis* outer membrane proteins

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Haemophilus influenzae type b (Hib) is still one of the leading causes of infection diseases among the poorest countries. Conjugate vaccines based on the Hib capsular polysaccharide or fragments thereof, coupled to a protein, demonstrated a high efficiency in infants. A number of scientific and technological questions, however, remained to be solved in order to achieve a worldwide vaccination and as a basis for future new conjugate vaccines.

In an effort to make a Hib vaccine, we have been tried for a number of years to develop a synthesis of a capsular polysaccharide fragment, good enough to be used in the preparation of a conjugate vaccine. The antigen issued from these efforts, was used in the present study as a conjugate to *Neisseria meningitidis* outer membrane proteins. The effect of the protein carrier on the immunogenicity of the conjugates was studied in SD rats that produce a strong anti-capsular polysaccharide antibody response. The avidity of the antibodies as well as their specificity for the capsular polysaccharide were also demonstrated, showing the potential of such conjugates as human vaccines.

C17. Glycolipids: metabolism/domains/signaling

C17.1

Sulfatide localization and metabolism in the β-cell line RIN-38; a potential model for studying the role of sulfatide in insulin processing and secretion

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Evidence are accumulating that the glycosphingolipid sulfatide (3′-sulfogalactosyl-ceramide) might play a role in insulin processing and secretion in the β-cells. To evaluate the possibility to use cultured β-cells for further studies concerning this interaction between sulfatide and insulin, sulfatide expression, localization and metabolism was investigated in the β-cell line RIN-38. Sulfatide was shown to be expressed by the RIN-38 cells and were accumulated in the secretory granules, the intracellular compartment where insulin is stored. The major biosynthetic pathway occurred by recycling back from the lysosome to the Golgi compartments, supporting a rapid metabolism of this glycosphingolipid. Sulfatide was also localized to the nucleus, a finding which has never been reported before. Moreover, sulfatide was accumulated in glycolipid enriched microdomains. These results support that the RIN-38 β-cell line might be a relevant model to further explore the role for sulfatide in insulin processing and secretion including the intracellular signaling events involved.

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C17.2

Overexpression of ganglioside sialidase in transgenic mice leads to non-insulin dependent diabetes mellitus

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We previously cloned a cDNA of human membrane-associated sialidase, which is specific for gangliosides and therefore seems to be essentially engaged in modification of cell surface gangliosides. For further understanding of physiological function of the sialidase, we have investigated the effect of overexpressing ganglioside sialidase in transgenic mice using the cDNA under the control of beta-actin promotor. Of the six lines of transgenic mice generated, the three lines developed fasting hyperglycemia only in male mice by 25 weeks. Impaired glucose tolerance and hyper-insulinemia were observed. Histological examinations of the pancreas of the transgenic mice showed immunohistochemically detectable insulin not only in islet but also in area other than typical islet, indicating overproduction of insulin in the transgenic pancreas. Insulin-stimulated phosphorylation of insulin receptor was reduced and the insulin responce of insulin receptor substrate I was less sensitive compared to those in wild type. Glycogen synthase activity tended to be decreased. The exogenous sialidase is likely to be involved in the impared insulin signal transduction through its phosphorylation. In conclusion, overexpression of the membrane sialidase gene led to non-insulin dependent diabetes mellitus, and the sialidase was found to play an important role in insulin signaling.

C17.3

Observation of topology of gangliosides in lipid monolayers by atomic force microscopy

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It is important to know the driving force for the formation of "raft" which is rich in the glycolipid in the cell surface. We have reported glycolipids showed the phase separation in phospholipid membranes from the analysis of surface pressure-molecule area isotherms [1]. In this study, such phase separation was observed using atomic force microscopy (AFM).
An air-water interface monolayer containing glycolipids and phospholipids was transferred on the mica substrate, and topology image of the mixed film was observed using AFM in air and in water. The sphingoglycolipid showed characteristic topology image dependeing on the membrane composition without perfectly mixing with the phospholipid. Especially, the clear domain of the glycolipid was observed when phospholipid (DOPC) with the unsaturated fatty acid was used as a lipid matrix. And, the domain formation was dependent on structure of the oligosaccharide, phase transition temperature of the lipid matrix, and surface pressure of monolayer. Therefore, raft structure was suggested to be controlled by the microenvironment and physico-chemical condition.

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C17.4

Apoptosis mediated by the Gb3/CD77 glycolipid antigen in Burkitt's lymphoma cells: comparison of the pathways triggered by anti-CD77 mAb and Verotoxin-1

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CD77, a B cell differentiation antigen, is a neutral glycosphingolipid called globotriasosylceramide (Gb3). Gb3 is also the receptor for most members of the Shiga toxin family, including verotoxin 1 (VT-1). Previously, we have shown that both anti-Gb3/CD77 mAb and VT-1 induce apoptosis of Burkitt's cells. In this report, we have compared the apoptotic pathways induced by these two molecules in native Gb3/CD77(+) Burkitt's cells and in cells which express it *de novo* after transfection of the recently cloned Gb3 synthase. VT-1 was found to induce apoptosis at a high level in all Gb3/CD77(+) BL cells, whether the glycolipid was expressed constitutively or after transfection of Gb3 synthase. In contrast, only native Gb3/CD77(+) cells were highly sensitive to the specific mAb. In both cell-types, Gb3 had the same ability to mediate the retrograde transport of VT-1 to the endoplasmic reticulum and was similarly located in the glycosphingolipid-rich microdomains. The Src-family tyrosine kinase Lyn was also similarly concentrated in these microdomains. However, co-immunoprecipitation experiments, using the apoptosis-inducing anti-Gb3/CD77 mAb, indicated that Gb3 and Lyn are associated in the glycosphingolipid-rich fractions of the native Gb3/CD77(+) cells, but hardly at all in the transfected cells. This observation suggests that the association of Gb3 with Lyn could be a crucial element in the anti-Gb3/CD77-induced apoptosis.

C17.5

Investigations on glucosylceramide synthase from rat liver and expression of the murine enzyme in *Sf*21 cells

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Murine glucosylceramide synthase (mGlc-T) was expressed in insect cells (*Spodoptera frugiperda, Sf*21) using the baculovirus expression vector system. The resulting recombinant mGlc-T (rmGlc-T) contained a His_6 tag and a TEV protease cleavage site at the N-terminus. The activities of Glc-T were determined in the homogenates of infected (rmGlc-T-*Sf*21) and uninfected control *Sf*21 cells, respectively, using an *in vitro* assay. The specific activities in the homogenates of rmGlc-T-*Sf*21 cells were up to 30 fold higher than those obtained with the homogenates of control cells. Analysis of the glycosphingolipid patterns after metabolic labelling with [^{14}C]serin showed that GlcCer synthesis was enhanced by a factor of >60 in infected cells. These results show that infection of *Sf*21 cells with the recombinant virus led to a strong overexpression of the rmGlc-T.
Preincubation with iodoacetamide resulted a strong concentration-dependent inhibition of Glc-T activity as well in rat liver Golgi preparations as in *Sf*21 and rmGlc-T-*Sf*21 homogenates. These results indicate that the glucosylceramide synthases of rat, mouse and *Spodoptera frugiperda* contain a catalytically essential cystein residues, most probably in their active sites.

C17.6

Glycosphingolipids are required for sorting of melanosomal proteins from the Golgi complex

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Glycosphingolipids are essential for embryonic development, but the mutant cell line GM95 lacks the ceramide-glucosyltransferase and survives without glycolipids. Parental melanoma cells (MEB4) are black, whereas the mutant cells are white. This difference is due to the mislocalisation of tyrosinase, the first enzyme involved in the biogenesis of the black pigment melanin. We provide evidence that glycolipids are involved in the sorting of melanosomal proteins (tyrosinase and tyrosinase related protein 1 (TRP1)). These melanosomal proteins both have in their cytoplasmic tail a signal recognized by an adaptor protein complex called AP3, involved in their targetting to melanosomes. Thus, glycolipids are required in the AP3 pathway to the melanosomes. In order to determine if this defect was specific for AP3, the influence of the lack of glycolipids on the AP1 pathway (from the Golgi to the lysosomes) was also investigated. The best documented proteins with an AP1 sorting signal are the mannose-6-phosphate receptors. The secretion of some lysosomal proteins transported via these is not affected in mutant cells, suggesting that the AP1 pathway is not affected when the cells have no glycolipids. In order to determine how glycolipids are specifically involved in the AP3 pathway, the binding of AP3 to membranes was investigated. The molecular mecanisms by which glycolipids are specifically involved in the AP3 pathway to the melanosomes remain to be determined.

C17.7

Dynamism of ganglioside GM3-enriched particles shed from GM3-enriched membrane microdomains

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Gangliosides, which form glycosphingolipid-enriched microdomains (GEMs/rafts), act as modulators for transmembrane signaling and as mediators for cellular interaction. GM3 (the simplest ganglioside), exogenously added in cell culture, can inhibit or stimulate cell growth. Gangliosides shed from tumor cells have been long suggested for their existence, and are thought to be involved in the escape of tumor cells from immune surveillance. However, there is no direct evidence so far for the dynamism of these microdomains. Here, we could for the first time grasp via immunofluorescence and immunoelectron microscopic techniques GEMs and their derivatives, extracellularily shed-out particles (50-200 nm, in diameter). This is the first direct evidence of the dynamism of the GM3-enriched particles, which are budding from the GEMs. The budding mechanism of GM3-enriched microdomains may be related to the characteristics of tumor cells, such as hyper-secretion, migration or metastasis.

C17.8

Characterization of glycolipid transfer proteins

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Background: Glycolipid transfer protein (GLTP) with a molecular weight of 22 kDa was initially identified as a cytosolic protein in bovine spleen. The protein facilitates in vitro intermembrane transfer of glycolipids: glucosylceramide (GlcCer), galactosylceramide (GalCer), and galactosyldiglyceride (GalDG). In the sequential glycosylation of glycosphingolipid biosynthesis, only the step of glucosylation of ceramide has been shown to occur on the cytoplasmic side of the Golgi membrane. It appears most likely that GLTP recognizes GlcCer and participates its intracellular traffic in vivo. In addition to the 22 kDa GLTP, recently the presence of another GLTP, a novel 44-kDa human protein, was revealed; 22 kDa and 44 kDa GLTPs are temporarily termed GLTP-1 and GLTP-2, respectively. GLTPs have a remarkable hydrophobic sequence, ALXWLKRGLXF, which is also found in orthologs in other mammals, flies and worms; however, the function of this sequence is so far unknown.
This study: We carried out several experiments to understand the molecular mechanisms of transfer activity and the exact physiological roles of GLTPs. RT-PCR analysis showed that transcriptions of both GLTP-1 and GLTP-2 are detected ubiquitously in human tissues although in varying amounts. Recombinant GST-GLTP-1 bound to GlcCer most preferentially among the lipids tested. When recombinant GFP-GLTP-1 was expressed in CHO cells, it was localized in cytosolic spaces in a soluble form.

C17.9

1,10-Phenantroline inhibits GPI anchor synthesis in mammals and yeast but not in the *Trypanosoma brucei* parasite

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Glycosylphosphatidylinositols (GPIs) anchor proteins to the plasma membrane of eukaryotes including *T. brucei*, yeast and mammals. We recently reported that 1,10-phenanthroline (PNT) inhibits the synthesis of mammalian GPI-anchored proteins by preventing the incorporation of phophoethanolamines (P-EthN) into GPI anchor precursors [1]. In mammals and probably yeast these reactions are carried out by three phosphoethanolamine transferases (PETs) whereas only one PET is likely to be present in *T. brucei*. In this study, we investigated whether PNT can block P-EthN additon to GPIs in membranes obtained from *T. brucei* and yeast. As in mammals, PNT prevented the synthesis of the mature GPI anchor as well as GPI-anchored proteins in yeast. Furthermore, PNT treatment resulted in the accumulation of a GPI anchor intermediate lacking P-EthN, suggesting that the yeast PET-1 is sensitive to the inhibitor. In contrast GPI anchor synthesis in *T. brucei* was not affected by PNT. Our results highlight the potential differences between *T. brucei* and mammalian/yeast PETs which may be exploted to develop antiparasitc drugs.

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C17.10

Biosynthesis of a hybrid glycolipid by a GalNAcT-1 activity isolated from guinea pig bone marrow

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Cell surface glycosphingolipid (GSL) antigens are classified in different categories according to the structure of their inner three sugars attached to ceramide. The internal third sugar in each case is attached as follows: Ganglio-, GalNAcβ1-4; Lacto-, GlcNAcβ1-3; Globo-, Galα1-3; Hybrid- GalNAcβ1-4 and GlcNAcβ1-3. Expression of the higher homologs of these GSLs on the surfaces of animal cells is tissue- and species-specific. The Hybrid GSLs are present on the surfaces of undifferentiated murine leukemic cells and absent on the surfaces of differentiated cells [1]. Functional role of this GSL on the cancer cell surface has yet to be determined [2]. The GalNAcT-1 has been isolated and purified from embryonic chicken brains [3] guinea pig bone marrow [4] and has been cloned [5]. The GalNAcT-1 from g.pig catalyzes the transfer of GalNAc from UDP-GalNAc to LcOse3ceramide (GlcNAcβ1-3Galβ1-4Glcβ1-1-ceramide). Among the potential acceptor tested only Lc2, GM3 and LcOse3Cer were active with this enzyme preparation. Using phenylglyoxal almost 100% activity was inhibited. However, 1,2-dichlorohexanedione and butadione inhibited about 60-80% under our present assay condition. Almost 90% of the terminal [^{3}H]GalNAc was cleaved when incubated for 60 h at 37° C with purified β-hexosaminidase isolated from papaya and clam. The structure of the hybrid GSL was established by mass spectrometry and immunostaining of the radioactive product against the antibodies raised against the authentic GSL and suggested as GalNAcβ1-4(GlcNAcβ1-3)Gal-Glc-cer. (Sup: CA-14764 to SB).

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C17.11

The ganglioside-specific sialidase of the plasma membrane co-fractionates with markers of lipid rafts

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Gangliosides situated on the external side of the plasma membrane are important modulators of cellular functions. In previous work on human neuroblastoma cells we had found that a ganglioside-specific sialidase activity of the plasma membrane was involved in the control of proliferation and differentiation by causing selective ganglioside desialylation. Recent reports have shown the enrichment of gangliosides in glycosphingolipid-containing membrane microdomains, called glycosignaling domain or rafts, which can be isolated due to their insolubility in Triton X-100 and flotation of the low-density fraction through a sucrose gradient. Assuming that the ganglioside sialidase should be in close proximity to its substrates in the same membrane, we investigated the possible association of the enzyme with detergent-insoluble glycolipid-containing microdomains in the neuroblastoma cell line SK-N-MC. The results show that the ganglioside sialidase co-distributes with ganglioside GM1 and other raft markers such as flotillin, src family kinases, and GPI-anchored proteins in a fraction containing about 2% of cellular protein. The association of the ganglioside sialidase with glycosphingolipid-enriched membrane fractions therefore further supports the suggestion of a role of this glycosidase in ganglioside-dependent signal transduction processes.

C17.12

Ganglioside GD1a signalling pathway through MAPK suppresses gelatinase B secretion by FBJ osteosarcoma cells

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We have shown that highly metastatic FBJ-LL cells lacked GD1a, in contrast with poorly metastatic FBJ-S1 cells, and the presence of ganglioside GD1a resulted in inactive cell motility and suppresion of cell adhesiveness to vitronectin [1]. Secretion of gelatinase B is regarded as one of the features of metastatic cells and this was also the case with FBJ cells [2]. Among the FBJ-related cells, secretion of gelatinase B was inversely proportional to the level of GD1a and pretreatment of cells with GD1a impaired gelatinase B production. Five minutes after incubation of cells with GD1a, intense phosphorylation of MAPK (Erks-1 and -2) was observed. This was preluded by phosphorylation of MEK (MAPKK) and followed by that of p90. Inhibition of MEK resulted in the failure to suppress gelatinase B secretion, indicating that signal transduction pathway through Erks is the main route by which ganglioside signal is transferred to the cell nucleus.

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C17.13

Expression of soluble cerebroside sulfotransferase in CHO cells

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Galactosylceramide and its sulfated derivative sulfatide are major components of the myelin membrane. Sulfatide is formed by the cerebroside sulfotransferase (CST, EC 2.8.2.11) in the Golgi apparatus and degraded in lysosomes by arylsulfatase A (ASA). Mutations in the ASA gene result in a severe lysosomal storage disease, metachromatic leukodystrophy (MLD). Inhibition of sulfatide synthesis potentially is a possible therapy for MLD. Therefore, we are interested to produce larger amounts of CST for structural and enzymatic characterisation and screening of CST inhibitors. We stably transfected CHO cells with a plasmid encoding protein A fusion protein with CST lacking its transmembrane domain (ProtA-CST). Thin layer chromatography and immunofluorescence revealed that ProtA-CST is active inside the CHO cells. ProtA-CST recovered from the culture supernatant and purified on IgG-sepharose was active in an in vitro sulfotransferase assay. Two putative N-glycosylation sites are present in both, human and murine CST (Asn-66 and Asn-312). To elucidate whether these sites are actually used and whether they are necessary for activity or stability of the enzyme we changed Asn-66 and Asn-312 to Gln separately and in combination in ProtA-CST as well as in the membrane bound enzyme. Analysis of these mutants revealed that both Asn residues are glycosylated. However, the unglycosylated enzymes remain active and the expression levels were similar to the wildtype protein.

C17.14

Apoptosis of Neuro2a cells induced by lysoglycosphingolipids: molecular cloning of lysoglycosphingolipid-generating enzyme

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Lysoglycosphingolipids (LGSLs), which lack the fatty acid moiety of glycosphingolipids (GSLs), are known to be accumulated in some variants of sphingolipidoses. We report here that LGSLs, which were prepared by bacterial LGSLs-generating enzyme (SCDase), induce apoptosis in mouse neuroblastoma Neuro2a cells. The viability and [^{3}H]thymidine incorporation of Neuro2a cells were strongly suppressed by the addition of LGSLs in a dose-dependent manner, while the parental GSLs had no effect. Chromatin condensation, intranucleosomal DNA fragmentation, phosphatidylserine externalization, and caspase-3 activation, which are typical features of apoptosis, were observed when the cells were cultured with 40-80 μM of LGSLs for 24-48 h in the presence of 5% FCS. The addition of an inhibitor of caspases, ZVAD-fmk, to the Neuro2a cell culture completely inhibited the elevation of caspase-3 activity but not the DNA fragmentation, suggesting that a caspase-3 independent signaling pathway is involved. This study may indicate that the accumulation of LGSLs, but not parental GSLs, triggers the apoptotic cascade in neuronal cells of patients with glycosphingolipidoses. The bacterial SCDase, consisting of 992 amino acids including a signal sequence of 35 residues, was cloned and functionally expressed in *E. coli*.

C17.15

Neutral ceramidase: missing link of sphingolipid signalling

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Sphingolipids and their metabolites have emerged as a new class of lipid modulators for various cell functions. Since sphingosine (Sph) is not generated from *de novo* synthesis, ceramidase, which hydrolyzes the *N*-acyl linkage of ceramide (Cer) to produce Sph, is crucial for the generation of Sph and possibly Sph-1-phosphate. Recently, we cloned novel neutral ceramidases from mouse and rat, which may be involved in sphingolipid-mediated signal transduction. The enzyme catalyzed not only the hydrolysis of Cer but also the synthesis of Cer in acyl-CoA-independent manner *in vitro* as well as *in vivo*. Expression of the enzyme differed depending on cell types, tissues and animal species; in rat liver the enzyme was distributed with endosome-like organelles in hepatocytes, while in rat kidney the enzyme was localized at apical membranes of proximal tubules, distal tubules and collecting ducts. Interestingly, the kidney enzyme was found to be enriched in raft microdomains with cholesterol and GM1 ganglioside.

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C18. Glycomics

C18.1

Structural analysis of N-glycans in *Caenorhabditis elegans*

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Caenorhabditis elegans, which is one of the simplest multicellular organisms, is a model animal suitable for functional glycomics. Though many glycogenes have been isolated from the worm and analyzed so far, actual glycan structures were unclear. To elucidate their biosynthetic pathways, we have attempted the structural analysis of N-glycans of *C. elegans*.

The N-glycans were liberated from the worms by hydrazinolysis, and tagged with a fluorophore, 2-aminopyridine. Ninety percent of the N-glycans was neutral and 10 % was acidic. HPLC-mapping and glycosidase digestions were used for characterizing structures of the neutral N-glycans. The most dominant structure was Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc (M3B). Oligomannose-type glycans of similar branching isomers to those of mammalians were also included with high content. There were core α1-6fucosylated M3B and GlcNAc-attached M3B at α3branch-mannose. These findings are consistent with other protostomes including insects. Some glycans had new structures containing β-galactoside but not usual complex- or hybrid-type. Although there were some glycogenes for synthesizing complex-type glycans in *C. elegans* genome, common complex type glycans were scarcely detected by HPLC-mapping.

C18.2

Glycopeptide-targeted Glycome Project: A progress in *Caenorhabditis elegans*

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Protein glycosylation is a dominant issue of proteomics, but so far no practical approach was available. Along with the concept glycomics, a novel procedure named "glyco-catch" was developed, by which glycoprotein genes, glycosylation sites can be determined as well as types of glycosylation (N-glycans, O-glycans, high-mannose type, complex type, etc.). The procedure is based on a conventional affinity technique using well-characterized lectins. As the first target of glycomics, glycoproteins secreted from the nematode *C. elegans* were enriched by using ConA (specific for high-mannose type N-glycans) and galectin LEC-6 (specific for complex type N-glycans containing *N*-acetyllactosamine or its linkage isomer lacto-*N*-biose) columns. Thus, adsorbed glycoproteins were digested with *Achromobacter* protease I, recaptured by the same lectin columns as above, separated by C1 reversed-phase HPLC, and subjected to protein sequencer analysis. As a result, >60 glycopeptides were successfully analyzed to identify >40 genes, mostly encoding digestive enzymes, stock/carrier proteins, and cell adhesion molecules, with a significant number of proteins with unknown functions. The proposed procedure meets a basic requirement for glycomics targeting many other organisms.

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C18.3

Glycomics of aberrant N-glycosylation in CDG syndrome by in-gel deglycosylation and MALDI-TOF MS mapping

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Inherited metabolic diseases based on defects in the N-glycosylation pathway of glycoconjugates are defined as Congenital Disorders of Glycosylation (CDG). A general feature of all different CDG types is hypoglycosylation of glycoproteins causing multisystem diseases of clinical significance, which can be correlated to the structures of N-glycan chains. In this contribution we describe a newly developed glycomics methodology for detection of aberrant structures in N-glycosylation from MALDI-TOF MS glycan maps obtained by enzymatic in-gel deglycosylation of 1- or 2D gel spots or by deglycosylation of wholesome human plasma. Potentials of this approach for CDG diagnosis will be discussed. Results obtained from MALDI-TOF MS differential N-glycan mapping including glycoproteins with different glycoforms deliver more detailed set of data contributing to diagnosis of complex diseases inborn errors of metabolism like different already known or unknown types of the CDG syndrom.

C18.4

Proteomic analysis of protein glycosylation

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Recent advances in the field of proteomics have made it possible for hundreds of proteins to be identified in a day. However, the characterisation of post-translational modifications is still typically achieved by slow "traditional" approaches, using relatively large amounts of purified proteins. We are interested in protein glycosylation, and have shown that underivatised glycans can be detected at the low fmol level, by LC-ESI-MS on a commercial ion-trap mass spectrometer. This is well within the range of protein levels that are typically present on 2D gels. We will show that information on the glycosylation of proteins can be achieved by methods that are compatible with a high-throughput proteomics environment.

Human plasma glycoproteins were separated by 2D gel electrophoresis, and either electroblotted onto PVDF or used directly. A number of protein spots were excised, digested with peptide N-glycosidase F, and the released N-glycans analysed by LC-ESI-MS. Good quality data can be obtained from even weakly stained spots. Data interpretation is assisted by the use of a database of glycan structures (GlycoSuiteDB - http://www.glycosuite.com). The deglycosylated protein can, if required, be further digested with trypsin for protein identification, and the whole procedure of spot excision, digestion and analysis is amenable to automation.

C19. Glycopathology/inflammation/infection

C19.1

Analysis of N-glycans of pathological tau: possible occurrence of aberrant processing of tau in Alzheimer's disease

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In a previous study, Wang *et al* (*Nature Med*, **2**:871-875, 1996) found (i) that abnormally hyperphosphorylated tau isolated from Alzheimer's disease (AD) brain as paired helical filaments (PHF)-tau and as cytosolic AD P-tau but not tau from normal brain were stained by lectins, and (ii) that on in vitro deglycosylation the PHF untwisted into sheets of thin straight filaments, suggesting that tau only in AD brains is glycosylated. To elucidate the primary structure of N-glycans, we comparatively analyzed the N-glycan structures obtained from PHF-tau and AD P-tau. More than half of N-glycans found in PHF-tau and AD P-tau were different. High mannose type sugar chains and truncated N-glycans were found in both taus in addition to a small amount of sialylated bi- and tri-antennary sugar chains. More truncated glycans were richer in PHF-tau than AD P-tau. This enrichment of more truncated glycans in PHF might be involved in promoting the assembly and or stabilizing the pathological fibrils in AD.

C19.2

A monoclonal antibody, MIN/3/60, that recognises sulpho-Lewis^x and sulpho-Lewis^a , detects a population of inflammatory cells

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Monoclonal antibodies directed to Lewis^x (Le^x) and related sequences have been invaluable in elucidating the roles of specific cell-surface oligosaccharides, and in identifying leukocyte subsets that are selectively recruited in inflammation. We now describe a monoclonal antibody (rat hybridoma MIN/3/60) raised to 3'-sulpho-Le^x, a carbohydrate sequence which, *in vitro,* is bound not only by the E-, L- and P-selectins, but also by the cysteine-rich domain of the macrophage endocytosis receptor. We observe that MIN/3/60 is bispecific, i.e. it binds sulpho-Le^a as well as sulpho-Le^x. Nevertheless, our exploratory studies reveal that it is a useful histochemical reagent when used in conjunction with a previously characterised monospecific anti-sulpho-Le^a *[Glycobiology 8:1237-1242 (1998)]*. The MIN/3/60 antibody reveals a population of infiltrating cells that express sulpho-Le^x, in the absence of sulpho-Le^a, in the inflamed synovium in rheumatoid arthritis. This is, to our knowledge, the first report of sulpho-Le^x on inflammatory cells. Further investigations are under way to determine details of the tissue distribution of the sulpho-Le^x-positive inflammatory cells, their possible interactions with antigen-presenting cells that express the macrophage receptor, and the mechanisms of their trafficking in inflamed tissues.

C19.3

Immunotoxicity of the extracellular water-soluble polysaccharide fraction released by *Candida albicans*, CAWS, in mice

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We have recently found that *Candida albicans* released a water-soluble polysaccharide fraction (CAWS) into synthetic medium. CAWS was mainly composed of mannoprotein and β-glucan, and in part, they were covalently bound. In this study, various activities of CAWS were observed. CAWS activated *Limulus* factor G, and showed lethal toxicity and vasculitis. Vasculitis induced CAWS was strain dependent: C3H/HeN; susceptible, DBA/2; resistant. Biological activities of CAWS in these strains of mice were composed. In vitro, CAWS inhibited the mitogen activity of lipopolysaccharide, and INF-γ production of Concanavalin A in both strains of mice in vitro. In vivo, CAWS induced (i) cell infiltration into peritoneal cavity and splenomegaly, (ii) INF-γ production by spleen cell culture, (iii) myeloperoxidase release by spleen cell culture. Especially, IFN-γ production by splenocytes from DBA/2 mice were higher than that from C3H/HeN. These facts suggested that CAWS showed various immunotoxicity and the difference of INF-γ production might be related to the susceptibility to vasculitis.

Reference

Uchiyama M *et al* (1999) *FEMS Immunol Med Microbiol* **24:**411-420

C19.4

Lectin staining gives many informations for postmortem diagnosis of patients with brain disorder

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We have been investigating glycoconjugate depositions in human brain from patients with or without brain disorder by means of lectin- and immunohistochemical techniques. In addition to the visualization of senile plaques, neurofibrillary tangles and Corpora amylacea with Con A, PSA, and/or GSAIB4 lectins, we found two types of glycoconjugate deposits, i.e. amorphous(AD) and spherical deposits(SD), in this study. The ADs which showed empty spaces by H.E. were detected in the white matter of the brain of patients with Down's syndrome, Alzheimer and non-Alzheimer type dementia, and/or patients with edematous brain by staining with Con A, PSA, UEA-I, GSAIB4 and/or DBA lectins, and an antibody against heparan sulfate. SDs were detected in the molecular layer of the dentate gyrus of the hippocampus formation of patients with schizophrenia and brain disorders by staining with UEA-I, GSAIB4 and/or DBA lectins, and an antibody against chondroitin sulfate. ADs may be a sequel to disfunction of the blood brainbarrier and SDs may cause dysfunction of the neuronal network in the dentate gyrus of the hippocampus which is closely linked with recognition and memory function. The results obtained in this study indicate that not only neuronal degeneration but also unusal glycometabolism in neurons and brainwhite matter may disturb the neuronal function and cause brain disorders.

C19.5

Septic shock induced by microbial glycans and non-steroidal inflammatory drugs

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Lethal toxicity was induced in mice by sequential administration of immunomodulating β-glucan from various fungi (3 times; day-5, -3, -1; ip or iv) and indomethacin (daily; day 0; po). Whole cell preparation of Gram-positive, Gram-negative bacteria, and fungi also show similar toxicity in combination with indomethacin (IND). Various non-steroidal anti-inflammatory drugs (NSAID) including aspirin showed similar toxicity. However, bacterial lipopolysaccharide (LPS) did not show such toxicity. In contrast, after IND administration, sensitivity of mice to LPS was significantly elevated and mice died by only 0.1 μg (iv). Accumulation of leukocytes in lung and liver, enhancement of TNF, IL-6, and MIP-2 syntheses, activation of neutrophil, hypothermia, hypoglycemia were accompanied. Antibiotic treatment prolongs survival of mice. From these facts, the lethal toxicity would be resembled with the septic shock and strongly related to the microbial translation and endotoxin absorption from the gut.

References

Yoshioka S *et al* (1998) *FEMS Immunol Med Microbiol* **21**:171-179
Takahashi H *et al* (2001) *FEMS Immunol Med Microbiol* (in press)

C19.6

Expression and secretion of CD43 by enterocyte-like Caco-2 cells *in vitro*

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Several glycoforms of CD43 are known to regulate cellular interactions in the immune system. Our previous studies showed that core 2 O-glycans were synthesized by human colon carcinoma Caco-2 cells after enterocytic differentiation. In the present study, we used a mAb 1D4 specific for CD43 bearing core 2 O-glycans and demonstrated that O-glycans attaching to CD43 produced by these enterocyte-like cells had core 2 structures. Furthermore, CD43 with core 2 O-glycans were stored in intracellular granules as shown by immunocytochemical studies and were secreted into culture media as indicated by ELISA. When enterocyte-like Caco-2 cells were incubated with LPS, CD43 mRNA levels were upregulated as shown by RT-PCR analysis. The amount of secreted CD43 with core 2 O-glycans also incresed. However, staining profiles of these cells incubated in the absense or presense of LPS with a polyclonal Ab against carboxy terminal domain of CD43 were very similar. Interesingly, 1D4-reactive granules prominently increased upon stimulation of these cells with LPS, suggesting that soluble forms of CD43 bearing core 2 O-glycans seemed to be rapidly produced and stored in quantities.

Reference

Amano J *et al* (1999) *J Biol Chem* **274:**21209

C19.7

Increased high molecular weight hyaluronan in urine of interstitial cystitis patients

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Interstitial cystitis (IC) is a chronic, painful bladder syndrome that is not life threatening, but it seriously affects the quality of life. A popular hypothesis about the etiology of IC is that the bladder glycosaminoglycans are defective allowing noxious urine components to penetrate and irritate the bladder. In previous studies, we showed that the level of urinary hyaluronan (HA) was higher in IC patients than healthy controls. We also demonstrated the presence of high concentrations of HA in the lamina propria of bladder. In this study, we analyzed the molecular size of HA in human bladder and urine of IC patients and controls. The HA isolated from bladder tissue had a molecular mass in excess of 40,000 daltons. In the case of urinary HA, while the bulk of the material was smaller than 10,000 daltons variable proportions were of higher mass. Statistically, a higher percent of HA in IC urine compared to control urine (3.76$\pm$4.23% versus 1.48 $\pm$1.71%, p =0.0459) had size characteristics similar to that of the bladder material. These findings suggest the possible leakage of bladder HA into urine in IC. Subepithelial HA is proposed to provide lubrication as the epithelium folds and flattens during voiding and bladder filling. Thus, the loss of bladder HA could be factor contributing to pain in IC. (Supported by USPHS grants DK57266 and DK 57281).

C19.8

Galectin-3 binds to gelatinase B and lactoferrin from human neutrophil leukocytes

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The mammalian lectin galectin-3 is a potent stimulus of oxidative burst in human neutrophils, but only after these cells have been primed by translocation from the blood stream into the inflammatory site. Priming involves the recruitment of the galectin-3 receptors, CD66a and CD66b from specific and gelatinase granules to the cell surface [1]. We have identified two new ligands for galectin-3 by affinity chromatography and MALDI-TOF; gelatinase B and lactoferrin. Gelatinase B is a matrix metalloproteinase, and it is likely that exocytosis of gelatinase granules is essential for migration of neutrophils through basement membranes. It may be speculated that binding of galectin-3 to gelatinase B and lactoferrin is augmenting the presentation of these proteins at the inflammatory site. By using confocal microscopy it was shown that galectin-3 may also be endocytosed by the neutrophils. The uptake occurred rapidly, and exogenously added galectin-3 was localized intracellular already after 15 min. The data suggest that galectin-3 may modulate inflammation through the direct interaction with neutrophils and/or their products. Furthermore, these studies will provide a basis for exploring the biomedical usefulness of galectin-3 and inhibitory ligand analogs.

Reference

Almkvist J *et al* (2001) *Infect Immun* **69:**832-837

C19.9

Studies on galectins and neutrophils

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The galectins comprise a group of β-galactoside-binding lectins with preferential affinity for poly-*N*-acetyl-lactosaminoglycans. We have studied the proinflammatory potential of galectin-1 and -3 in their interaction with human neutrophils, primarily with regard to activation of the superoxide-producing NADPH-oxidase and binding to neutrophil glycoconjugates. The galectin-induced NADPH-oxidase activation was assessed in peripheral blood neutrophils as well as in primed cells. The galectins were able to activate the NADPH-oxidase provided that the cells had been primed by *in vivo* extravasation or *in vitro* pretreatment with lipopolysaccharide (LPS) or chemoattractants. We suggest that the mechanism behind induction of the galectin-responsiveness lies at the level of granule mobilisation and upregulation of galectin receptors to the cell surface. By subcellular fractionation of peripheral blood neutrophils followed by galectin overlay several galectin-1 binding epitopes were identified in the neutrophil gelatinase granules and secretory vesicles and out of the galectin-3 binding proteins in the specific and gelatinase granules, CD66a and CD66b were the most probable receptor candidates.

References

Karlsson A *et al* (1998) *Blood* **91**:3430-3438
Feuk-Lagerstedt E *et al* (1999) *J Immunol* **163**:5592-5598
Almkvist J *et al* (2001) *Infect Immun* **69**:832-837

C19.10

Change in glycosylation of matrix glycoprotein in liver cirrhosis alters ligand binding in matrix remodeling

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Vitronectins (VN) are multifunctional adhesive glycoproteins present in plasma and the extracellular matrix of most tissues. In chronic liver diseases, the collagen-binding VN in plasma correlates with certain fibrous markers [1]. We found that the change in glycosylation after partial hepatectomy enhanced the collagen binding of VN [2]. To elucidate the glycan modulation, changes of VN in cirrhosis were studied. Plasma concentrations of VN declined in liver cirrhosis (LC) to 2/3 that in normal plasma, but the VN that is active in collagen binding increased in cirrhotic plasma whereas most VN is inactive in normal (N-) plasma. In contrast, purified LC-VN exhibited attenuated binding to type I, IV and V collagen compared to N-VN. Lectin reactivities and carbohydrate analyses of LC-VN revealed that branching, fucosylation, and sialylation of N-glycans were elevated compared to those of N-VN. The attenuated collagen binding of purified LC-VN can be attributed to the changes of glycosylation, e.g., increased sialylation of LC-VN in comparison to N-VN, because decreased sialylation enhanced the collagen binding of VN produced during liver regeneration [2]. The increase in active VN in LC plasma may contribute to the matrix incorporation of VN and tissue fibrosis.

References

[1] Yamada S *et al* (1996) *Clin Chem Acta* **252**:95-103
[2] Uchibori-Iwaki H *et al* (2000) *Glycobiology* **10**:865-874

C19.11

O-Glycosylation in neurons in Alzheimer's disease, indicating reactive plasticity

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Reactive plasticity, including axonal and dendritic sprouting and reactive synaptogenesis, has been proposed to contribute to the pathogenesis of several neurological disorders. This work was aimed at identifying the possible role of protein glycosylation in the brain from patients with Alzheimer's disease (AD), using lectin histochemistry, as determinants of reactive plasticity. Results indicate an increase in the production of cryptic O-glycosydically linked proteins (Neu5Acα2,6-Galβ1,3GalNAcα1,O Ser/Thr) in neuritic sprouting in AD brains as determined by positive labeling with *Amaranthus leucocarpus* (T-antigen specific) and *Macrobrachium rosenbergii* (Neu5,9Ac_2 specific) lectins. Immunohistochemistry indicated that lectin-staining was specific for the synaptic sprouting process (meganeurites) in AD. These results were confirmed using anti-synaptophysin and anti-GAP 43 antibodies, which recognized meganeurites and dystrophic neurites around amyloid-β deposits. In normal control brains labeling with the aforementioned lectins was restricted to microvessels. Control experiments with neuraminidase treated brain samples revealed positivity to PNA lectin. Our results suggest specific O-glycosylation patterns of proteins, closely related to neuronal plasticity in AD. (Financed in part by CONACYT and PAPIIT-UNAM, Mexico).

C19.12

Increased O-glycoprotein expression on CD4+ T cells in human tuberculosis

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The protective immune response against *M. tuberculosis* is maintained by CD4+ T cells. *Amaranthus leucocarpus* lectin (ALL) recognizes an O-glycosylated structure in human CD4+ T cells. The aim of this study was to determine the role of CD4+ ALL+ T cells in pulmonary tuberculosis (TBP). We purified CD4+ T cells from patients with TBP: 20 PPD+ (*Group 1*) and 12 healthy volunteers (*Group 2*). The purified cells were analyzed with indirect immunofluorescense with CD25, CD124, CD57 and ALL-FITC. Our results indicated that in *Group 1,* 5% of the CD4+ T cells were CD124+, and 2% were CD124+ ALL+; in *Group 2,* 9% of the CD4+ T cells were CD124+, and 1% were CD124+ ALL+. We found that in *Group 1,* 6% of the CD4+ T cells were CD25+, and 2% were CD25+ ALL+; in *Group 2,* 14% of the CD4+ T cells were CD25+ and 2% were CD25+ ALL+. The percentage of CD57+ on CD4+ T cells were 9% in *Group 1;* and 3% were CD57+ ALL+; in *Group 2,* 3% were CD57+ and <1% were also CD57+ ALL+. We observed in *Group 1* that 7% of the CD4+ T cells were ALL+; in *Group 2,* 3% of the CD4+ T cells were ALL+. To evaluate the expression of the O-glycoprotein on CD4+ T cells after antigenic stimulation, CD4+ T cells were co-cultured with its homologous macrophages in presence of mycobacterial antigen. After 24-48 hrs of specific stimulation we observed an increment in the percentage of CD4+ ALL+ T cells in patients with TBP. This results suggest the participation of a specific cellular subset CD4+ ALL+ in the immune response in human tuberculosis. (This work was financed by CONACYT and PAPIIT-UNAM).

C19.13

Differences on the expression of an O-glycosylprotein on CD4+ T cells, compared with phenotypic cell surface markers, on different atopic diseases

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The approach of the atopic diseases with cell surface markers has been important to explain their immunopathology. In our laboratory we have identified a surface O-glycoprotein (ALL-R) which could be a good marker to study these processes.
Objective: To identify differences on the expression of ALL-R on different cell surface markers either on CD4+ T cells and antigen presenting cells, on several atopic diseases.
Methods: NA, Non Atopic subjects (n=4); NR, Non symptomatic atopic Rhinitic patients (n=4); SR, Symptomatic atopic Rhinitic patients (n=4); MA, Mild atopic Asthmatics (n=5); SA, Severe Acute atopic Asthmatics (n=5); AD, Atopic Dermatitic patients (n=5); CD, Contact Dermatitic patients (n=3). All the subjects except NA and CD, were positive to skin prick test to *Der. Pteronyssinus*, seric IgE > 300 UI/ml. The PBMC were isolated and labeled with direct immunofluorescent antibodies and were acquired by FACS.
Results: CD4+/ALL-R+: AD (10%)vs NA (0.01%), $p<0.05$; CD4+/CD30+: AD (22%) vs. CD (3%), $p<0.05$; CD4+/CD62L: AD (29%) vs. CD (56%), $p<0.02$; SA (11%) vs. MA (76%), $p<0.02$; CD4+/CD124+: AD (19%) vs. CD (3%), $p<0.04$; CD4+/CD152+: AD (15%) vs. CD (1%), $p<0.008$. The results of APC were as follows: CD14+/CD80+: MA (17%) vs. NA (2%) $p<0.001$. The differences on the expression of cell surface markers analyzed in this work suggest that Atopic Dermatitis is the disease with the most expression of this O-glycoprotein, therefore with the most unbalanced Th2 profile. (Financed in part by CONACyT and PAPIIT-UNAM).

C19.14

TNFα increases the expression of glycosyltransferases involved in the biosynthesis of sialylated and/or sulfated Lewis x epitopes in the human bronchial mucosa

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There is increasing evidence that inflammation may affect glycosylation of various glycoproteins. The present study reports the effect of TNFα, a proinflammatory cytokine, on the glycosyltransferases of the human bronchial mucosa, responsible for the biosynthesis of Lewix x epitope and of its sialylated and/or sulfated derivatives which are expressed in human bronchial mucins. Fragments of macroscopically normal human bronchial mucosa were exposed to TNFα at a concentration of 20 ng/ml. TNFα was shown to increase α1,3-fucosyltransferase activity as well as expression of the two α1,3-fucosyltransferase genes expressed in the human airway, *FUT3* and *FUT4.* It had no influence on α1,2-fucosyltransferase activity or *FUT2* expression. It also increased α2,3-sialyltransferase activity, the expression of genes *ST3Gal-III* and, more importantly, *ST3Gal-IV.* These results are consistent with the observation of oversialylation and increased expression sialyl-Lewis x epitopes on human airway mucins secreted by patients with severe lung infection such as those with cystic fibrosis whose airways are colonized by *Pseudomonas aeruginosa.* However other cytokines may also be involved in this process.

C19.15

Recognition of Lewis x derivatives present on mucins by flagellar components of *Pseudomonas aeruginosa*

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*Pseudomonas aerugi*nosa (*P.a.*) binds to human respiratory mucins by mechanisms involving flagellar-receptor interactions. The adhesion of *P.a.* strain PAK is mediated by the flagellar cap protein, FliD. Two distinct types of FliD proteins have been identified in *P.a.*, A type found in strain PAK and B type in strain PAO1. In the present work, studies performed with the *P.a.* B type strain PAO1 indicate that both the FliD protein and flagellin are involved in the binding to respiratory mucins. It was previously demonstrated that *P.a.* recognizes Le^x derivatives found on human respiratory mucins. The aim of the present work was therefore to determine whether these epitopes are receptors for FliD proteins and flagellin. The results obtained by both flow cytometry using fluorescent glycoconjugates, and a microplate adhesion assay indicate that the FliD protein of strain PAO1 is involved in the binding of glycoconjugates bearing Le^x or sialyl-Le^x determinants, while the binding of flagellin is restricted to the glycoconjugate bearing Le^x glycotope. In contrast, the type A cap protein of *P.a.* strain PAK is not involved in the binding to these glycotopes. This study demonstrates a clear association between a specific *P.a.* adhesin and a specific mucin epitope.

C19.16

Structural studies of the exopolysaccharide amylovoran

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Fire blight is a bacterial disease caused by *Erwinia amylovora* on apple and pear trees, hreatening complete orchards. Other Rosales host plants have been described. The bacterial coat can play an important role in pathogenicty and host range. The exopolysaccharide amylovoran is the main compound of the *E. amylovora* coat. Nimtz and coworkers have shown for one Malaceae isolate that the exopolysaccharide consists of a pentasaccharide repeating unit. It was suggested by these authors that another repeating unit of the exopolysaccharide could be an hexasaccharide, where a β-D-glucopyranosyl residue is added to the basic pentasaccharide repeating unit. With NMR we have fully proven the structure of this hexasaccharide repeating unit. Therefore we developed a new method to identify 1-6 linkages, based on selective excited TOCSY, edited GHSQC for methylene protons and chemical shift considerations.

Reference

Nimtz M *et al* (1996) *Carbohydr Res* **287:**59-76

C19.17

Significance of galactosylation of IgG3 cryoglobulins in the renal pathogenicity

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The cryoglobulin activity associated with murine IgG3 has been shown to play a significant role in the development of murine lupus-like glomerulonephritis. A fraction, but not all, IgG3 monoclonal antibodies are capable of inducing a severe acute lupus-like glomerulonephritis as a result of direct localization of IgG3 cryoglobulins, suggesting the importance of qualitative features of cryoglobulins in their nephritogenic activities. Here we show a remarkable difference in the renal pathogenicity of two murine IgG3 monoclonal cryoglobulins, identical in the amino acid sequence of their heavy and light chains, but different in galactosylation pattern of the oligosaccharide side chains because of the synthesis in different myeloma cells. The antibody lacking the capacity of inducing severe glomerulonephritis showed an increased proportion of galactosylated heavy chains. Changes in conformation, as revealed by gel filtration analysis, reduced cryoglobulin activity, and accelerated clearance could account for the lack of the renal pathogenicity of the more galactosylated variant. This observation directly demonstrates the role of IgG galactosylation in the pathogenic potential of cryoglobulins.

C19.18

Role of lipid portions of glycosphingolipids in adhesion of *Helicobacter pylori*

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Recognition of cell surface carbohydrates plays an essential role in colonization and initiation of infection of bacteria. It has been suggested that one putative group of receptor molecules of *H. pylori* is glycosphingolipids expressed on epithelial surface of the gastric mucosa, such as sulfatide and GM3. However, roles of the carbohydrate region and the lipid portion of glycosphingolipids are not well understood. In the present study, *H. pylori* adhesion to glycosphingolipids was compared to that to neoglycolipids or polyacrylamide-derivatives that carried the oligosaccharides corresponding to the glycosphingolipids. *H. pylori* clearly adhered to immobilized sulfatide, GM3, galactosylceramide and lactosylceramide. In contrast, *H. pylori* did not adhere to polyacrylamides conjugated with β-galactose, lactose, 3'-sialyllactose or 3'-sulfo-β-galactose, each of which corresponds to the carbohydrate residue of galactosylceramide, lactosylceramide, GM3, or sulfatide. The neoglycolipids composed of phosphatidylethanolamine and the carbohydrate corresponding to the glycosphingolipids did not serve as ligands for *H. pylori*. In addition, ceramide itself and sphingomyelin did not serve as the ligands. These results strongly suggest that both carbohydrate and ceramide are essential for recognition of glycosphingolipids by *H. pylori*.

C19.19

Characterization of oligosaccharides and activity of IgG rheumatoid factor

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It has been shown that rheumatoid produces abundant aggregates of IgG and rheumatoid factors (RF). RF activity has been detected in all of the immunoglobulin isotypes, but IgG with RF activity (IgGRF) is considered to be more important in the pathology of rheumatoid arthritis (RA). To elucidate the relation between IgGRF immune complex formation and oligosaccharides in IgGRF, three IgGRF fractions and an IgG fraction without RF activity (non-RF IgG) were prepared from sera of RA patients using an IgG-Sepharose column, and their oligosaccharide structures and RF activities were characterized. All the IgG samples contained a series of biantennary complex-type oligosaccharides, but the levels of both sialylation and galactosylation of all IgGRFs were lower than those of non-RF IgG from RA patients and total IgG from healthy individuals. The decrease in galactosylation and sialylation of oligosaccharides in IgGRF well correlated with the increase in RF activity. These data suggest that the lower the level of galactosylation and sialylation of IgGRF oligosaccharides, the higher the RF activity. In addition, analyses of the RF activity by means of surface plasmon resonance detection indicate that IgGRF forms immune complexes more rapidly at lower temperature. This nature is different from those of the other RFs (IgARF and IgMRF).

C19.20

Role of IgG1 oligosaccharide in binding of IgG1 to FcγRIII

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In murine macrophages, FcγRIII has been known to mediate phagocytosis of opsonized particles and to be a sole receptor mediating IgG1-dependent phagocytosis. It has been reported that the oligosaccharide moiety of IgG molecule was indispensable for the appearance of the effector function of an antibody such as the binding to macrophage Fc receptor and the clearance of immune complexes from circulation. In order to clarify the role of IgG1 oligosaccharides in IgG1-dependent phagocytosis *via* FcγRIII, we analyzed the oligosaccharide of the following two types of IgG1 anti-red blood cell (RBC) mAb: mAb 31-9D, which exhibits a weak binding to FcγRIII and agglutinates RBC, and mAb 105-2H, which exhibits a strong binding to FcγRIII and induces erythrophagocytosis. The structures of oligosaccharides were analyzed with HPLC in conjugation with sequential exo-glycosidase digestion, after the oligosaccharides had been released by hydrazinolysis and labeled with *p*-aminobenzoic acid ethyl ester. Both mAbs contained the same series of complex-type biantennary oligosaccharide. However, the incidence of agalactosylated oligosaccharides with a non-reducing terminal GlcNAc in mAb 105-2H was higher than that in mAb 31-9D. These results suggest that galactosylation of IgG1 is involved in the IgG1-dependent phagocytosis and the binding of IgG1 to FcγRIII.

C19.21

Prognostic potential of *Maackia amurensis* leukoagglutinin for histochemical analysis of human gastric cancers

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Enhanced sialylation has been considered to be important in tumor metastasis and progression. In this study, we performed histochemical analysis of human gastric cancer tissues using *Maackia amurensis* leukoagglutinin (MAL), a sialic acid-binding lectin, and examined the potential of the lectin for determining prognosis in patients with gastric cancer. All tissue samples prepared from 71 patients showed positive staining with MAL in all or part of the tumor region, but not in the corresponding non-cancerous region. The distribution of the values of the MAL-staining indices (MI), the frequency of stained cells among tumor cells, showed two distinct populations with a cutoff level of 40%. High MI levels (>40%) were significantly frequent in cases of lymph node metastasis, lymphatic vessel invasion, and venous invasion as well as in advanced tumors. In analysis of survival, patients having a tumor with a high MI level showed a significantly poorer prognosis than those with a low MI level (<40%). Furthermore, multivariate analysis showed that a high MI is an independent factor for shorter survival together with the depth of and the venous invasions. These results suggest that MAL would be an effective tool for determining prognosis in patients with gastric cancer.

C19.22

Polymorphisms of two *fucosyltransferase* genes (*Lewis* and *Secretor* genes) involving type I Lewis antigens are associated with the presence of *anti-Helicobacter pylori* IgG antibody

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Helicobacter pylori attach to the gastric mucosa with adhesin, which binds to Lewis b or H type I carbohydrate structures. The *Secretor* (*Se*) gene and *Lewis* (*Le*) gene are involved in type I Lewis antigen synthesis. The present study was performed to investigate the possibility that *Se* and *Le* gene polymorphisms alter the risk of *H. pylori* infection. Two hundred thirty-nine participants were genotyped for *Se* and *Le* and tested for the presence of anti-*H. pylori* IgG antibodies. The *H. pylori* infection rate was positively associated with the number of *Se* alleles and negatively associated with the number of *Le* alleles. Among the three groups divided according to these genotypes and the tendency to *H. pylori* infection, the high and moderate risk groups exhibited infection rates 10 times (OR=10.33, 95%CI 3.16~33.80) and 3 times (OR=3.30, 95%CI 1.40~7.78) higher than the low risk group. Using the normal gastric mucosa from 60 gastric cancer patients, we assessed immunohistochemically whether type I Lewis antigen expression depended on the Se and Le genotypes. Immunohistochemical analysis supported the finding that *Se* and *Le* genotypes affected the expression of *H. pylori* adhesin ligands. *Se* and *Le* genotype affects susceptibility to *H. pylori* infection.

C20. Glycoproteins: metabolism/ folding/trafficking/sorting/quality control

C20.1

Clusterin containing high-mannose type glycans is vectorically secreted from MDCK cells through the lectin activity of VIP36

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Vesicular integral protein of 36 kDa (VIP36) has lectin activity recognizing high-mannose type glycans with $Man_{7\text{-}9}GlcNAc_2$. To elucidate the functional role of VIP36 in relation to intracellular transport of *N*-linked glycoproteins, we investigated the intracellular transport of the major secretory glycoprotein, clusterin, in relation to VIP36 in MDCK cells. We found that clusterin secreted from MDCK cells bound to VIP36 and Endo H treatment of clusterin diminished its binding to VIP36. These results suggested that clusterin contained at least one high-mannose type glycan. Using the VIP36 overexpressed clone and D131N-mutant VIP36 which lacks the lectin activity overexpressed clone, we compared the secretion of clusterin from apical side of the cells. In VIP36 overexpressed clone, the secretion of clusterin in apical side was sharply increased. On the other hand, the overexpression of D131N-mutant VIP36 did not affect the secretion of clusterin. Furthermore, electron microscopic analysis using anti-VIP36 antibody showed the distribution of VIP36 from ER to plasma membrane throughout the secretary pathway in MDCK cells. These results indicated that VIP36 plays important roles in intracellular transport of glycoproteins through its lectin activity from ER to cell surface.

Reference

Hara-Kuge S *et al* (1999) *Glycobiology* **9**:833-839

C20.2

3D reconstruction of lipoprotein-containing structures at the ER-cisGolgi interface of rat hepatocytes

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The hepatocyte ER and Golgi process lipoprotein particles (LPs) which because of their size (35-75 nm) are considered supramolecular structures. To elucidate LP transport (Tx) across the sER-cisGolgi interface, the relative topography of the LP-filled sER, -cis-Golgi and -intermediate structures was analyzed in 60+serial TEM Golgi fields, from Long Evans rats. The Golgi fields were captured in single or panoramic micrographs, photographed through 3-9 consecutive 60-90 nm sections. Selected serials were computer reconstructed by (1) scanning the sections into Adobe Photoshop of a PowerMacintosh computer; (2) contouring membrane-bound structures within the scanned images; (3) concatenating and volume rendering the files in Synu on an SGI and (4) stereorendering the stacked files in Photoshop. The serials/constructions showed LP-filled tubules to project up from the cis-most Golgi element and towards and into LP-filled loops of sER. LP-filled -tubules and -sER loops were separated by a few nanometers only. While clusters of potential intermediate transport vesicles (each 100-300 nm diameter) which contain LPs were identified within the larger ER-cisGolgi interface, the LPs contained within such vesicles were smaller, less dense and associated with invaginations of the vesicular membrane. We propose that the vesicles are sites for LP processing/degradation, rather than LP transport, and that LP-transport across the ER-cis Golgi interface occurs by saccular maturation or by direct fusion between the closely apposed subcompartments of the ER and cis-Golgi.

C20.3

LacdiNAc sequence (GalNAcβ1→4GlcNAcβ1→) specific for secretory glycoproteins in MDCK cells

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High mannose type glycans are important for intracellular transport of glycoproteins in MDCK cells by binding to VIP36, an intracellular lectin. To determine whether some complex type sugar chains are also associated with specific localization of glycoproteins, secretory and surface glycoproteins of apical and basolateral sides were separated from metabolically labeled MDCK cells ([^{3}H]-glucosamine), and their *N*-linked glycans were analyzed. The [^{3}H]-oligosaccharides released from secretory and surface glycoproteins of apical side had about twice radioactivity than those of basolateral side, but their structures of both sides were equal to each other. Their predominant structures consisted of high mannose type glycans and biantennary complex type glycans with or without bisecting GlcNAc. The most interesting carbohydrate features were LacdiNAc sequences on the non-reducing terminal. Over 20% of oligosaccharides derived from secretory glycoproteins of both sides contained LacdiNAc, in contrast oligosaccharides from both surface glycoproteins did not contain this moiety. Moreover, the LacdiNAc could not be observed in oligosaccharides containing bisecting GlcNAc. These results indicate that the LacdiNAc is synthesized under strict substrate specificities of various glycosyltransferases and this moiety might play an important role for secretory pathway of MDCK cells.

Reference

Hara-Kuge S *et al* (1999) *Glycobiology* **9**:833-839

C20.4

Sorting of proteoglycans in the secretory pathway of epithelial MDCK cells

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In epithelial MDCK cells, secretion is directed towards the opposite apical and basolateral surfaces. While most heparan sulfate proteoglycans (PGs) are secreted basolaterally, chondroitin sulfate (CS) PGs are sorted and secreted only into the apical medium. Xyloside-based CS chains were also secreted apically, indicating that the sorting information may reside in the glycosamino-glycan (GAG) portion [1]. The CSPG is recognised by antibodies to Versican [2]. Our current investigations are addressing if other portions of the PG than the GAG chains are involved in sorting of CSPG into the pathway from the trans-Golgi network to the apical surface of MDCK cells.

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C20.5

Proteoglycan expression and enzyme secretion in polarized human colon carcinoma Caco-2 cells

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Caco-2 cells cultured on semipermeable supports acquire the morphology and functional abilities of normal enterocytes after 10-12 days. Treatment with 0.1 mM benzyl xyloside stimulates secretion of ^{35}S labeled macromolecules into both the apical and basolateral media, most obviously basolaterally. This is observed for both undifferentiated (3-4 days) and differentiated (10-12 days) cells, and the xyloside-based glycosaminoglycans (GAGs) are exclusively of the chondroitin sulfate (CS) type. Heparan sulfate proteoglycan levels were not affected by xyloside treatment. The level of trypsin-like activity was measured in the apical and basolateral media by use of chromogenic substrates. Xyloside treatment increased the level of enzyme activity in the basolateral medium, suggesting that proteoglycans may play an important role in regulation of secretion of such enzymes in polarized epithelial cells.

C20.6

Nuclear entry of lactosylated poly-L-lysine/cDNA complex in CF airway epithelial cells

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Lactosylated poly-L-lysine (LacpK)/cDNA complex was translocated into the nucleus of CF airway epithelial cells intact [1]. The nuclear entry was further investigated using WGA which binds to GlcNAc residues present on nuclear pore complex (NPC) proteins and blocks transport through the NPC [2]. CF airway epithelial cells were incubated with FITC-WGA for 1 h and subsequently transfected [1]. Confocal microscopy of cells demonstrated that WGA inhibited the nuclear entry of LacpK/cDNA complex. WGA was internalized and accumulated around the nucleus within 1 h and remained there up to 6 h. When cells pretreated with WGA were transfected with LacpK/ rhodamine-cDNA complex, no nuclear accumulation of complex was observed. Transfected cells not pretreated with WGA showed nuclear accumulation of complex. Thus nuclear entry of the complex is inhibited by WGA. A nuclear entry mechanism has been described for adenovirus [3]. A similar process could apply for LacpK/cDNA complex with the exception that galectins may provide the docking site close to NPC and facilitate binding to intranuclear lectins. These initial studies showed that LacpK/cDNA complex may enter the nucleus through the NPC. (Supported by NIH R21-DK55610; Ter Meulen Fund).

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C20.7

Identification of structural signals conferring Golgi-localisation to Man_9-mannosidase from human kidney

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Man_9-mannosidase, an α-1,2-specific exo-enzyme involved in the early pathway of N-linked oligosaccharide processing, has been cloned from a human kidney cDNA library [1]. The enzyme is a type II transmembrane N-glycoprotein containing a short cytoplasmic peptide tail, a 24 amino acid trans-membrane domain and a large ~59 kDa catalytic domain in the lumen [2]. Transfection of Cos 1 cells with a vector construct encoding for the complete Man_9-mannosidase polypeptide, resulted in the over-expression of a catalytically active and Golgi-resident enzyme. In order to identify structural signals involved in Golgi-targetting, a series of Man_9-mannosidase deletion and chimaeric protein constructs was synthesized and their subcellular localisation analysed by immunofluorescence microscopy after expression in COS1-cells. The results of these studies showed that information directing Golgi-specific localisation is likely to reside in the cytosolic and the trans-membrane rather than in the luminal domain of the Man_9-mannosidase polypeptide.

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C20.8

A mannose-trimmed N-glycan is implicated in degradation of a truncated ribophorin I in a glycosylation-defective cell line

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We investigated the role of N-glycans in the process of glycoprotein degradation using a previously described glycosylation-defective cell line, MadIA214. The HA epitope-tagged soluble variant of ribophorin I, RI_{332}-3HA, was expressed as a substrate for ER-associated degradation (ERAD) in this cell line. N-linked glycans were indeed shown important for proteasomal degradation of RI_{332} in HeLa and CHO cells where ER mannosidase I could trigger the targeting of the protein to degradation. Glycoproteins in MadIA214 cells carry truncated $Glc_1Man_5GlcNAc_2$ N-glycans which interfere with protein folding. This oligomannoside structure is deprived of the specific mannose which is removed by ER mannosidase I. However, ERAD of RI_{332}-3HA occurred rapidly, was proteasome-mediated, and α1,2-mannosidase inhibitors also stabilised RI_{332}-3HA in MadIA214 cells. The major N-glycan structure bound to total glycoproteins shortly after synthesis was the Man_5 derivatives. In contrast, RI_{332}-3HA carried only Glc_1Man_5 and Man_4 glycans, which were replaced by Man_4 and then Man_3 when proteasomal degradation was inhibited. An α1,2-mannosidase activity which is different from the one exhibited by ER mannosidase I is therefore involved in the ERAD of RI_{332}-3HA in the MadIA214 glycosylation-defective cell line.

C21. Glycosidases/gene organization/expression

C21.1

Purification and substrate specificity of endo-β-mannosidase from lily flowers

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Endo-β-mannosidase from lily (*Lilium longiflorum* Thunb) flowers is a new type endoglycosidase that acts on the Manβ1-4GlcNAc linkage of the core structure of N-linked sugar chains. We have purified the enzyme using the following separation methods; ammonium sulfate precipitation, DEAE-Sephacel, Superdex 200, hydroxyapatite, Mono Q, and Poros HS chromatography. From 1200 g of flowers 36 μg of the enzyme was obtained. The purified enzyme gave a single band by Native-PAGE and a single peak by gel chromatography. Three bands were detected by SDS-PAGE of the enzyme indicating that it was a heterotrimer consisting of subunits with molecular masses of 42, 31, and 28 kDa.

The substrate supecificity of the purified enzyme was investigated using several pyridylaminated (PA-) sugar chains. The enzyme preferentially hydrolyzed $Man_nMan\alpha1$-6Manβ1-4GlcNAcβ1-4GlcNAc-PA (n=0-2). But sugar chains containing Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc-PA was not hydrolyzed. These results indicate that the endo-β-mannosidase hydrolyzed highmannose type sugar chains without the Manα1-3 residue linked to Manβ1-4GlcNAc.

Reference

A Sasaki *et al* (1999) *J Biochem* **125**:363-367

C21.2

Isolation, characterization and molecular cloning of β-*N*-acetyl-hexosaminidase from *Aspergillus oryzae* CCM1066

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β-Hexosaminidase was purified from the culture medium of the collection strain *Aspergillus oryzae* CCM1066 to homogeneity. This enzyme is a glycoprotein, and the N-glycans of high mannose type can be removed under nondenaturing conditions. The deglycosylation leads to a significant increase of the activity of the enzyme due to a decrease in K_M. Using N-terminal and internal peptide sequences, the enzyme was cloned and sequenced. The sequence confirms a high degree of similarity with other enzymes of glycohydrolase family 20.

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C21.3

Expression of recombinant polysialyltransferase ST8SiaIV in insect cells using a baculovirus expression system

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Polysialic acid (PSA, poly-α2,8-*N*-acetylneuraminic acid) is a linear carbohydrate structure of α2,8-linked sialic acid residues. It can be found as a specific and highly regulated posttranslational modification of the neuronal cell adhesion molecule (NCAM). The presence of PSA on NCAM has been shown to modulate cell-cell interactions and to be involved in neural development, neural regeneration and plastic processes in the vertebrate brain. The polysialylation of NCAM is catalyzed by two different enzymes, the polysialyltransferases ST8SiaII (STX) and ST8SiaIV (PST), that are individually able to generate polysialylated NCAM. For both enzymes a second enzymatic activity could be shown, their ability to catalyze autopolysialylation. Recombinant expression of active ST8SiaIV has so far only been described in mammalian host cells. We established an baculovirus expression system for high level expression of recombinant ST8SiaIV in insect cells. A recombinant baculovirus was constructed to express soluble and secreted ST8SiaIV. Recombinant enzyme was then produced from baculovirus-infected *Spodoptera frugiperda* (Sf9) cells. The protein was identified by Western Blot analysis. Enzymatic activity could be detected in *in vitro* activity assays.

C21.4

Glycosylhydrolases activities in the transformed ginseng tissue

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The glucosylhydrolases are crucial for a number of major biological pathways (cellular signalling, biosynthesis and degradation of structural and storage polysaccharides, host-pathogen interactions). The levels of activity of β-D-glucosidase, α-D-mannosidase, β- and α-D-galactosidase, 1,3-β-D-glucanase, 1,6-β-D-glucanase, 1,4-β-D-glucanase, 1,4-α-D-glucanase were measured in *Panax ginseng* root and callus cultures. Significantly increased levels of activity of β-D-galactosidase, α-D-galactosidase and 1,3-β-D-glucanase were detected in transformed by the *rolC*-gene cells, compared with non-transformed cells, while the levels of activity of the other enzymes were unchanged. Surprisingly, a low level of β-D-glucosidase was detected both in transgenic and non-transgenic tissues. These data, as well as the gel-permeation experiments revealed that transformation of ginseng cells by the *rolC* gene could significantly affect activity of some carbohydrases and production of their molecular forms.

C21.5

Correction of mouse GM1-gangliosidosis by the human β-galactosidase transgene and generation of knockout/transgenic mice expressing mutant human β-galactosidases

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GM1-gangliosidosis is a progressive neurological disease caused by a deficiency of lysosomal β-galactosidase (β-Gal) and is classified into three clinical forms: infantile, juvenile and adult. We recently generated β-Gal knockout (KO) mice as an authentic model of GM1-gangliosidosis. In this study, we introduced the wild-type human β-Gal transgene into β-Gal KO mice in order to rescue their GM1-gangliosidosis phenotypes. We also introduced into β-Gal KO mice the mutant human β-Gal transgenes encoding R201C and I51T amino acid sub-stitutions, common for juvenile and adult GM1-gangliosidoses, respectively. Transgenes contained the β-actin promoter driving wild-type, R201C or I51T β-Gal cDNA expression. Transgenic founders were crossed with β-Gal KO mice to produce mice defective for native β-Gal but expressing the wild-type human β-Gal. As expected, the wild-type β-human Gal transgene rescued the mouse GM1-gangliosidosis. KO/transgenic mice expressing mutant human β-Gal might therefore be useful for the studies of pathogenesis and treatment of GM1-gangliosidosis with residual enzyme activities.

C21.6

Synthesis of α-D-Glc*p*-(1→2)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→3)-α-D-Man*p*-O$(CH_2)_8COOCH_3$ tetrasaccharide for α-glucosidase I activity

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α-Glucosidase I inhibitors have been shown to display antiviral activities [1]. Therefore, monitoring the activity of glucosidase I and the effect of inhibition of the enzyme, has provided information on the mechanism of glycoprotein formation and function in both normal and cancerous cells [2]. [^{14}C]- or [^{3}H]-glucose-labelled oligosaccharide substrates from microsomes or cell culture [3,4] have been utilized for α-glucosidase I assay with limited kinetic information. We have reported spectrophotometric assays for glucosidase I activity using α-D-Glc*p*-(1→2)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-O$(CH_2)_8COOCH_3$ trisaccharide [5]. We have also reported the synthesis of the trisaccharide and the product, disaccharide α-D-Glc*p*-(1→3)-α-D-Glc*p*-O$(CH_2)_8COOCH_3$ and α-D-Glc*p*-O$(CH_2)_8COOCH_3$ along with glucosidase I activity [6]. Here, we report the retrosynthesis and the glucosidase I activity of the tetrasaccharide α-D-Glc*p*-(1→2)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→3)-α-D-Man*p*-O$(CH_2)_8COOCH_3$ as part of the high mannose type natural glucan.

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C21.7

Mammalian chitinases: Characterization of functional properties and possible biological functions

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Next to cellulose, chitin is the most abundant glycopolymer on earth, being present as a structural component in coatings of many species, ranging from fungi to arthropods. Chitinases (EC 3.2.1.14) are ubiquitous chitin-fragmenting hydrolases that have been identified in several organisms. Until a few years ago it was generally assumed that man lacks the ability to produce a functional chitinase. Our observation of a markedly elevated chitotriosidase activity in plasma of Gaucher disease patients formed the basis of the identification of chitotriosidase, the first human chitinase that is specifically expressed by phagocytes. A frequently encountered deficiency in chitotriosidase stimulated us to investigate the possibility for compensatory other chitinases in mammals. A study in rodents led to the identification of a second mammalian chitinolytic enzyme that is also present in humans. The properties of this acidic mammalian chitinase (AMCase), are reported, and the possible implications of its existence are discussed.

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C21.8

Biochemical studies on recombinant Neu2, a novel human cytosolic sialidase

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Using a sequence homology-based approach we have identified a novel human gene, encoding Neu2, a cytosolic sialidase of 380 amino acids. Here we describe the basic biochemical characteristics and substrate specificity features of recombinant Neu2 produced by *E.coli* cells and purified to homogeneity by FPLC. The enzyme is highly stable and cleaves, besides MU-NeuAc, also sialyllactose, sialo-glycoproteins and gangliosides, with a pH optimum of 5.6. The used sialo-glycoproteins appeared to be far, the poorest substrates. The enzyme cleaves the α2-3 sialosyl linkage the best, followed by the α2-8 linkage present in GD1b, instead the α2-6 linkage is cleaved only in sialo-glycoprotein substrate. The greatest affinity and activity on α2-3 linkages can be seen with the gangliosides GD1a and GT1b, then followed by α-2,3 sialyl-lactose, the sialo-glycoproteins and GM3. The enzyme was activated by detergent only in the presence of gangliosides substrates, demonstrating that mixed micelles more efficiently affected than pure micelles. This gives rise to the notion that the enzyme recognises, besides the sialosyl linkage and the part(s) of the substrate molecule to which sialic acid is linked, but also the supramolecular organization of sialoglycoconjugates of an amphiphylic nature, like gangliosides.

C21.9

Cloning and characterisation of the hetero-dimeric glucosidase II-complex from human liver

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Glucosidase II is a heterodimeric protein complex consisting of a large α-subunit (~112 kDa) and a small β-subunit (~60 kDa). The enzyme which removes the two α1,3-linked glucose residues from Glc_2-Man_9-$GlcNAc_2$, has been cloned from a human liver cDNA library using standard techniques [1]. Several independent α- and β-subunit-specific λ-clones were isolated; the sequences were found to differ by distinct in-frame insertions/deletions, pointing to alternative splicing of transcripts. Northern blot analysis of different human tissues revealed a ~4.4 kb and ~2.4 kb transcript for the α- and the β-subunit, respectively, consistent with their full length cDNA. Transfection of COS1-cells with an α-subunit-specific vector construct resulted in the over-expression of a catalytically inactive ~112 kDa protein, whereas a greater than 4-fold increase in glucosidase II activity was detected when the α-subunit was co-expressed together with the β-subunit. Since previous studies had shown that a purified glucosidase II preparation lacking the β-subunit, was catalytically active [2], we conclude from these observations that the β-subunit is involved in α-subunit maturation rather than being required for catalysis once the α-subunit has aquired its active conformation.

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C21.10

α-Galactosidase and α-*N*-acetylgalactosaminidase of *Aspergillus niger*

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The screening of viable cultures of approximately 1400 strains of microorganisms permit to select micromycetes *Aspergillus niger*, producing into the culture liquid α-galactosidase and α-*N*-acetyl-galactosaminidase. So far as some others glycosidases were presented, the culture liquid was fractionated by ammonium sulphate (30 and 90% of saturation). Partially purified α-galactosidase and α-*N*-acetyl-galactosaminidase were obtained. Both enzymes are thermostable. Its incubation at 40°C during 90 min only insignificantly (5%) decrease the ability to hydrolyze synthetic substrates: *p*-nitrophenyl α-D-galactoside and *p*-nitrophenyl *N*-acetylgalactosaminide. α-Galactosidase and α-*N*-acetylgalactosaminidase completely retain catalytic activity in the pH values between 6.0 and 7.0 during 24 h incubation. A number of carbohydrates, guanidine derivatives and bovine blood were studied as inducers of α-galactosidase and α-*N*-acetylgalactoaminidase synthesis. It was shown that growing *Aspergillus niger* on fluid medium, containing glucosamine, galactosamine or galactose simultaniously with bovine blood lead to increase of α-*N*-acetylgalactosaminidase activity in 1.5 times. Some guanidine derivatives (guanidine carbonate, nitroguanidizone dimethylbenzaldehyde and nitroaminoguanizone salicilic aldehyde) increased the activity of α-galactosidase in 2 times and α-*N*-acetylgalactosaminidase – in 1.5 times.

C21.11

Molecular evolution of endoglycoceramidase

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Endoglycoceramidase [1] (EGCase, also known as ceramide glycanase [2]) is an enzyme capable of cleaving the glycosidic linkage between oligosaccharides and ceramides of various glycosphingolipids. We found that the Asn-Glu-Pro (NEP) sequence, the active site of family A cellulases (endo-1,4-β-glucanases), was conserved in two microbial EGCases [3,4]. Replacement of the Glu residue in the NEP sequence with Gln or Asp by site-directed mutagenesis caused a significant loss of enzymatic activity in EGCases but did not affect the expression of the EGCase proteins [4]. Recently, we cloned the eukaryotic EGCase from the jellyfish, *Cyanea nozakii* [5] and found that the NEP sequence was conserved in not only procaryotic EGCase but also the eukaryotic enzyme. These findings clearly indicate that the NEP sequence is the active site of EGCases, in which the Glu residue plays an important role in the catalytic reaction, possibly in the same manner as in endo-1,4-β-glucanase. The findings in this study also suggest that EGCase and cellulase are derived from the same ancestral gene.

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C21.12

Molecular cloning of cDNA encoding α-*N*-acetylgalactosaminidase from *Acremonium* sp. and its expression in yeast

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α-*N*-Acetylgalactosaminidase (α-GalNAc-ase) is an exoglycosidase specific for the hydrolysis of terminal α-linked *N*-acetylgalactosamine in various sugar chains. The cDNA, *nagA*, encoding α-GalNAc-ase from *Acremonium* sp. was cloned, sequenced, and expressed in yeast *Saccharomyces cerevisiae*. The *nagA* contains an open reading frame which encodes for 547 amino acid residues including 21 residues of a signal peptide in its N-terminal. The calculated molecular mass of mature protein from the deduced amino acid sequence of *nagA* is 57,260 Da, which corresponds to the value obtained from SDS-PAGE of native and recombinant enzymes treated with endo-β-*N*-acetyl-glucosaminidase H. Enzymatic properties of the recombinant NagA were almost the same as those of the *Acremonium* enzyme. We found that the enzyme had transglycosylation activity. The amino acid sequence of NagA showed significant similarity to those of eucaryotic α-GalNAc-ases and α-galactosidases (α-Gal-ases), particularly α-Gal-ase A (AglA) from *Aspergillus niger*. Phylogenetic analysis revealed that NagA does not belong to the cluster of vertebrate α-GalNAc-ase and α-Gal-ase but forms another cluster with AglA and yeast α-Gal-ases. Thus, the evolutionary origin of the fungal α-GalNAc-ase is suggested to be different from that of vertebrate α-GalNAc-ase.

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C21.13

Sequence analysis of glycosylhydrolases: β-fructosidase and α-galactosidase superfamilies

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Glycosidases are a widespread group of enzymes that hydrolyze the glycosidic bonds between two carbohydrate residues. Currently, several thousand sequences of the proteins are known. They are grouped into 81 families (http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html) on the basis of sequences similarity. Glycosidases with β-fructosidase activities belong to two families. GH32 family includes sucrases, various fructanases, and some fructosyltransferases; GH68 family mainly contains bacterial levansucrases. According to our data, enzymes of these two families, as well as α-L-arabinases and β-xylosidases (GH43 and GH62 families), show significant sequence similarity and, therefore, form a superfamily. Sequence analysis has allowed us to distinguish several subfamilies in each family. Surprisingly, in the case of GH43 family no correlation has been found between the subfamily membership and the origin of the proteins, suggesting that horizontal transfer plays a certain role in their evolution. GH4, GH27, and GH36 families include α-galactosidases. The latter two families, having sequences similarity, form a superfamily. GH27 family includes, for the most part, eukaryotic glycosidases and can be divided into three subfamilies containing the enzymes of plant, animal, and fungal origins. GH36 family consists of two subfamilies, one of which includes glycosidases from Gram-positive bacteria and fungi and the other one contains enzymes from Gram-negative bacteria. Enzymes of GH27 and GH36 families show distant relationship with glycosidases of GH31 family as well.

C21.14

Glycosidases: Automatic docking of *N*-acetylglucosamine β(1-4) oligomers

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Hen egg white lysozyme was taken as a model glycosidase in order to test the accuracy in determining the active site and the ligand accommodation of Autodock version 3.0, an Automatic Docking program. One crystal structure for the wild-type enzyme was selected from the PDB databank. This structure was obtained by co-crystallization with tri-*N*-acetyl-chitotriose, an enzyme inhibitor. Different oligomers of *N*-acetylglucosamine were built and then minimized in vacuo. The resulting structures where further pre-processed using AutodockTools: partial atomic charges were set using the Gasteiger method and full ligand flexibility was allowed. Dockings were performed using the Lamarckian genetic algorithm (LGA). In each test, 100 docking were performed, each having a population of 100 individuals. An interesting result is the difference between the calculated position of chitotriose and the experimental one: while the active site is formed by six subsites capable of binding one *N*-acetylglucosamine monomer each, the calculated and experimental ligands share only one of them, extending from there in opposite directions. One possible explanation is given taking into account the catalytic role of the subsites and the interactions between the different components of the complex. A detailed analysis of the results for the other ligands is also reported.

C22. Glycosyltransferases/gene organization/expression

C22.1

Purification and characterization of chick corneal β-glucuronyltransferase

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β-Glucuronyltransferase (GlcA-T), which transfers glucuronic acid (GlcA) from UDP-GlcA to GalNAc at the non-reducing end of chondro-pentasaccharide-PA (pyridylamino), GalNAcβ1-(4GlcAβ1-3GalNAcβ1)$_2$-PA, was purified 339-fold with a 11.0% yield from 2-day-old chick corneas by chromatography on DEAE-Sepharose, WGA-agarose, Heparin-Sepharose, and, 1st and 2nd UDP-GlcA-agarose (in the presence of Gal) columns. The activity was detected by fluorescence of the PA residue of the product. The enzyme was not bound to several chromatography columns except for Heparin-Sepharose and UDP-GlcA-agarose (with Gal). The purified enzyme has a optimum pH of 7.0 (HEPES buffer), and much higher activity toward chondro-hepta-saccharide-PA than toward the chondro-pentasaccharide-PA, but no activity toward *p*-nitrophenyl-β-GalNAc. The enzyme activity was almost completely inhibited by GalNAc (20 mM). SDS-polyacrylamide gel electrophoresis of the purified enzyme still showed four main protein bands at 41 kDa and below 14.4 kDa. The amino acid sequence will be determined for the band of 41 kDa. It seems that the enzyme is involved in the elongation of the chondroitin sulfate chain *in vivo*.

C22.2

α2,6-Sialylation control in β-galactoside α2,6-sialyltransferase (ST6Gal.I)-transfected colon cancer cell lines

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Among the most prominent colon cancer associated glycosylation changes there are an elevation of ST6Gal.I enzyme activity [1] and an increased α2,6-sialylation of cell membranes [2]; both are correlated with a worse prognosis. We have frequently observed a discrepancy between the ST6Gal.I level of a colon cancer sample or cell line and the relative level of SNA reactivity [3]. In this study, we have investigated quantitatively the biosynthesis of the sialyl-α2,6-lactosaminyl structure in two colon cancer cell types expressing the ST6Gal.I cDNA under the control of a constitutive promoter. We reached the following conclusions: (1) the mRNA level, determined by competitive RT-PCR, is not constant in the same cell line; (2) the level of enzyme activity only partially correlates with the mRNA level; (3) the percentage of Galβ1,4GlcNAc termini which remain unsubstituted is very high, even in the presence of a very high ST6Gal.I activity, and is strongly dependent on the cell type. Together, our results indicate that postranscriptional and postranslational mechanisms play a pivotal role in the control of α2,6-sialylation in colon cancer cells.

References

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C22.3

Trafficking and localisation of a resident *medial*-Golgi glycosyltransferase

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The mechanisms that determine the steady-state distribution of resident Golgi glycosylation enzymes may include aggregation, lipid mediated sorting and/or retrograde transport. We have compared the aggregation state of medial and late Golgi glycosyltransferases and have demonstrated that at pH 6.5 two medial Golgi enzymes exist as large molecular weight complexes in contrast to two late acting Golgi enzymes that are present as monomers and dimers [1]. Analysis of *N*-acetylglucosaminyltransferase I (GnTI) chimeras indicated that the formation of high Mr complexes is dependent on the GnTI luminal sequences. The localisation of *medial*-Golgi enzymes is likely to involve retrograde transport pathways, however, the trafficking of these resident enzymes through the Golgi stack is unclear. To investigate if GnTI is transported to the late Golgi, a modified GnTI bearing an N-glycan site on the C-terminus was constructed. The modified GnTI was functionally active *in vivo*, localised to the Golgi stack, and was N-glycosylated with sialylated complex N-glycan chains. Brefeldin A treatment of transfected CHO cells resulted in a dramatic reduction in sialylation of glycosylated GnTI. Our data imply that GnTI is transported to the *trans*-Golgi network, suggesting that GnTI continuously recycles from the late Golgi. A model will be presented based on aggregation and retrograde transport to account for the asymmetric distribution of GnTI within the Golgi stack.

Reference

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C22.4

Conserved peptide motifs of glycosyltransferases

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Glycosyltransferases (GTs) constitute a large group of enzymes that are involved in the biosynthesis of oligosaccharides and polysaccharides. These molecules of fascinating diversity mediate a wide range of functions, from structure and storage to specific signalling in eukaryotes. These enzymes transfer sugar residues from an activated donor sub-strate, usually a nucleotide-sugar, to an acceptor that may be a growing oligosaccharide, a protein or a lipid.
With the huge amount of data coming from the large-scale sequencing project, the need for appropriate computational tools to retrieve all sequences belonging to one defined GT group is becoming more and more important. We have concentrated our efforts on some of the major GT classes that participate in the synthesis of complex glycans in glycoproteins. We also included in this study, enzymes that use a dolichol-phosphate, either as a sugar donor or as an acceptor. For each GT class examined, the methodology used was: (1) to retrieve all relevant sequences from the databases, (2) to compare protein sequences to define groups of homologous proteins and (3) to determine group-specific peptide motifs that could constitute a "signature". These motifs can be used to scan various genomes to search for all possible GT sequences sharing a similar signature. In addition, the use of the sensitive Hydrophobic Cluster Analysis (HCA) method allowed the detection of conserved structural regions in GT groups showing no or very limited sequence homology.

C22.5

Towards understanding of EPS from structure to formation: characterization of the GTFs from LAB

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In the dairy industry, microbial exopolysacharides (EPSs) produced by lactic acid bacteria (LAB) are used in situ to improve the texture of fermented dairy products such as yoghurt or cheese. In addition, it has been suggested these EPSs may confer health benefits.
Gene clusters and structures of EPSs from different food-grade bacteria, *Streptococcus* and *Lactobacillus*, have now been identified. It has led to the building up of a toolbox of almost 20 different glycosyltransferases (GTFs).
To date, very little is known about the catalytic mechanisms of these enzymes and the molecular basis, which accounts for donor and acceptor specificity, are poorly understood. We are studying the enzymatic characteristics of purified, recombinant GTFs from these very gene clusters. A picture from a gene structure to an EPS structure will be presented.
With the discovery of novel enzymes from microbial sources and advances in the genetic engineering of GTFs, production of EPSs exhibiting desired properties is now feasible.

Reference

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C22.6

Differential expression of mRNAs for sialyltransferase isoenzymes induced in the hippocampus of mouse following kindled-seizures

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Sialic acids play important roles in various biological functions. To know which sialylation of glycoprotein and glycolipids affects neural plasticity, we first assayed differential expressions of sialyltransferase isoenzymes (STs) participating in amygdaloid-kindling among models of neural plasticity. Seven STs; ST3Gal I-IV, ST8Sia IV, ST6Gal I, and ST6GalNAc II, expressed in the adult mouse hippocampus by RT-PCR and showed diverse localization patterns in the hippocampus on in situ hybridization, suggesting that the individual cells expressed relevant ST. Furthermore, upregulation of ST3Gal IV and ST6GalNAc II mRNAs and downregulation of ST3Gal I and ST8Sia IV mRNAs were observed in the hippocampus following kindled-seizures. In addition, in the thalamus, ST3Gal IV mRNA increased gradually during kindling epileptogenesis. These results indicate that ST expressions are regulated by physiological activity and may play a role in neural plasticity.

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C22.7

Substrate specificities of "brain-type" β-1,4-galactosyltransferase

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GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)(GlcNAcβ1-4)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc (BA-2) was specifically and dominantly expressed in mouse brain. We previously reported that β-1,4-galactosyltransferase (β4GalT) expressed in mouse brain can not transfer galactose to BA-2. In this present study, substrate specificities of β4GalT in mouse brain were analyzed by using glycopeptides as substrates, and effects of sugar structures on the brain type β4GalT activity was investigated. β4GalT in mouse liver could transfer galactose to a glycopeptide with agalactobiantennary N-glycan and to a glycopeptide with BA-2. β4GalT in mouse brain could transfer galactose to a glycopeptide with agalactobiantennary N-glycan, however could not transfer galactose to a glycopeptide with BA-2. These results were the same as analyzed by using PA-N-glycans as substrates. We therefore investigated the effects of BA-2 on the galactosyltransferase activities in mouse brain using agalacto-biantennary N-glycan as substrate. The transferase activity of β4GalT in brain was inhibited by BA-2, however, that in liver was not inhibited by BA-2. The BA-2 acted as an inhibitor against "brain type" β4GalT with a *Ki* value of 0.29 mM.

Reference

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C22.8

Distribution of Tamm-Horsfall glycoprotein and expression of glycosyltransferases involved in the Sd^a-antigen biosynthesis in human kidney

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Tamm-Horsfall glycoprotein (THP) is a GPI-anchored protein prevalently exposed at the luminal face of tubular cells of distal nephron that is largely excreted in normal urine (50-100 mg per day). We have found that a proteolytic cleavage at the juxtamembrane ectodomain of GPI-anchored THP on human kidney is responsible for the urinary excretion [1]. More than 90% of Caucasian individuals express the Sd^a antigen at the non-reducing end of THP N-glycans. We have studied the distribution of THP, the activity of Sd^a-βGalNAc-transferase as well as the α2,3-sialyltransferase and α2,6-sialyltransferase expres-sion in the renal cortex and outer medulla of two Sd(a+) individuals. The relative proportion of THP in the outer medulla and in cortex has been found to be 1.2% and 0.41% of total proteins, respectively. Similarly, the activity of Sd^a-βGalNAc-transferase was significantly higher in the outer medulla. While in the two kidney regions no difference in the α2,3-sialyltransferase expression was found, the α2,6-sialyltransferase was well detectable in the outer medulla but not in cortex. The THP predominance in the outer medulla is consistent with microscopic analysis indicating that it is mainly present in the thick ascending limb of Henle. The different distribution of glycosyl-transferases in the two regions in which THP is synthesised suggests a microheterogeneity in the carbohydrate assembly of THP, namely in respect to the Sd^a antigen expression.

Reference

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C22.9

The acceptor and site specificity of human α1,3-fucosyltransferase IX

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Human α1,3-fucosyltransferase IX (Fuc-TIX) is expressed tissue specifically in brain, stomach, spleen and peripheral blood leukocytes [1]. It forms the Lewis x (CD15) epitope (Galβ1-4(Fucα1-3)GlcNAc). This study examines further the detailed acceptor specificity of Fuc-TIX, and its site specificity on polylactosamines. We found that although Fuc-TIX preferentially fucosylates the distal lactosamine unit of a linear polylactosamine chain [2], the addition of a single GlcNAcβ1-3 residue on the distal side of the reacting unit does not affect reactivity. Furthermore, on sialylated polylactos-amines the site-specificity is reversed: Fuc-TIX preferentially fucosylates the two most reducing-end lactosamine units of Neu5Acα2-3(Galβ1-4GlcNAc)$_4$. Therefore we conclude that in addition to forming distal Lewis x epitopes, Fuc-TIX may also have a role in the biosynthesis of internally fucosylated polylactosamines.

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C22.10

Transcriptional regulation of the hST6Gal I gene during differentiation of the HL-60 cell line

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We studied the regulation of the β-galactoside α2,6-sialyltransferase (hST6Gal I) gene during HL-60 cell. During HL-60 cell line differentiation, cell surface levels of α2,6-sialic acids expression decreased. Activities of hST6Gal I and levels of hST6Gal I mRNA dramatically decreased. Using RT-PCR, we found the major hST6Gal I mRNA isoform in HL-60 cells contains 5'-untranslated exons Y and Z. These results suggest that the expression of cell surface α2,6-sialic acids is controlled at the mRNA level, which is regulated by P3 promoter. Using a luciferase assay, we identified a functional DNA portion that confers an enhancer, located at nt -317 to -174 within the P3 promoter. This element contains two sequences similar to Sp1 and one sequence similar to Oct-1 recognition motifs. Site-directed mutagenesis of Sp1 and Oct-1 sites showed that two Sp1 motifs and one Oct-1 motif are essential for transcriptional activity in HL-60 cells. Enhancer activity is suppressed during HL-60 cell differentiation induced with DMSO. These results suggest that GC-box and octamer sequence may play a critical role in the transcriptional regulation of the hST6Gal I gene during HL-60 cell differentiation.

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C22.11

Genomics of mouse and human sialyltransferase genes

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So far, 18 species of sialyltransferases have been cloned from mouse and their enzymatic properties have been characterized. The cloned sialyltransferases can be classified into four families according to the carbohydrate linkages they synthesize, *i.e.*, the β-galactoside α2,3-sialyltransferase family (ST3Gal I-VI), the β-galactoside α2,6-sialyltransferase family (ST6Gal I), the GalNAc α2,6-sialyltransferase family (ST6GalNAc I-VI), and the α2,8-sialyltransferase family (ST8Sia I-V). We have performed genomic structural analysis of mouse and human sialyltransferase genes in the last few years, and found that several sets of sialyltransferase genes share similar genomic structures. These enzymes also show similar substrate specificities and amino acid sequence similarities, suggesting that these genes arose from a common ancestral gene through gene duplication. In fact, some of these genes are located close to each other on the same chromosome (human ST6GalNAc I and II genes on Chr17 and human ST6GalNAc IV and VI genes on Chr9), although mechanisms of transcriptional regulation of these genes are not necessarily similar. Based on these observations, sialyltransferases can be further classified into subfamilies from an evolutional standpoint. These results will be useful for the analysis of molecular evolution of sialyltransferase genes.

C22.12

Expression and purification of a recombinant soluble β1-4 galactosyltransferase fusion protein from *Escherichia coli*

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Investigations concerning the expression of β4galactosyltransferases of human or bovine origin in *Escherichia coli* have demonstrated the formation of inclusion bodies yielding inactive enzyme [1].We here report on a novel vector (*pLGalTΔ38*) for the periplasmic expression of a soluble human β4Gal-T1 fusion protein in *E. coli*. The fusion protein contains the soluble, catalytic C-terminal domain from human β1-4GalT1 in a N-terminal fusion with the pre-pro-peptide of a lipase from *Staphylococcus hyicus*. Most interestingly, the formation of inclusion bodies was not observed. The expression yielded high levels of active enzyme, 30 mU/mg protein in the crude extract, which are 1000 fold higher compared to other expression systems in *E. coli*. The recombinant β4Gal-T1 was purified to near homogeneity in four purification steps by anion exchange chromatography, IMAC, cation exchange chromatography, and size exclusion chromatography. The N-terminal fusion protein was cleaved off during IMAC, most probably via catalysis through the immobilized metal ions. After IMAC purification a specific activity of 0.5 U/mg was achieved.

Reference

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C22.13

Regulation of PNA binding in activated peripheral CD8+ T cells

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Changes in PNA binding during the course of T cell intrathymic maturation, as well as after activation in the periphery, have been well documented. The physiological importance of these changes has been documented in a ST3GalI knock-out mouse, which showed that constitutive expression of PNA binding sites leads to a marked increase in apoptosis of peripheral CD8+ cells [1]. In the thymus, mature T cells are PNA low when compared to immature cells, a process reported to be controlled by the levels of ST3GalI [1,2]. In contrast, the mechanism for the increased levels of PNA binding after activation of peripheral T cells has not been elucidated. Previous studies have shown an increase in neuraminidase activity following T cell activation. However, a specific neuraminidase with appropriate specificity has not been identified [3]. In order to examine the basis of the underlying changes in PNA binding following activation of peripheral CD8+ cells, we have analyzed the expression levels of the mRNAs of several glycosyltransferases and neuraminidases before and after activation of CD8+ cells. Initial studies, using real-time PCR and DNA microarray technology [4], revealed differences in the expression levels of ST3GalI and Neu3 consistent with their role in producing increased PNA binding following cell activation.

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C22.14

Evolutionary conservation of substrate specificity of a novel putative orthologous pair of UDP-*N*-acetylgalactosamine: polypeptide *N*-acetylgalactosaminyltransferases, human GalNAc-T11 and *Drosophila l(2)35Aa (dGalNAc-T1)*

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Putative murine and human orthologs of the *Drosophila melanogaster* polypeptide GalNAc-transferase gene *l(2)35Aa* (*d*GalNAc-T1) were cloned and expressed (Schwientek *et al*, unpublished). The mammalian genes encoded polypeptide GalNAc-transferases with identical properties, and were designated GalNAc-T11. GalNAc-T11 exhibits a unique expression pattern, that includes selective and high expression in kidney. Human GalNAc-T11 and the *Drosophila d*GalNAc-T1 displayed unique and characteristic substrate specificities by comparative *in vitro* analysis with a panel of peptide substrates with multiple potential acceptor sites derived primarily from mucin tandem repeat sequences. These results support the proposed orthologous relationship between mammalian GalNAc-T11 and *Drosophila d*GalNAc-T1. The observed conservation of orthologous subfamilies of GalNAc-transferase isoforms with unique catalytic properties from fly to man strongly supports the hypothesis that evolution of this large gene family was driven by a profound functional requirement for substrate specificity rather than need for functional redundancy.

C22.15

A microarray for glycosyltransferases gene expression profiling

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Changes in the expression of specific glycoconjugates influence many biological events, such as embryogenesis, oncogenesis, cell activation and differentiation. In order to understand the biosynthetic basis for such changes, it is important to evaluate the expression of glycosyltransferase genes responsible for their synthesis. Classical techniques such as Northern blot, semi-quantitative RT-PCR and differential display have been used to understand these phenomena, but are limiting with respect to the number of genes that can be evaluated. We have begun to create a focused DNA microarray that will in principle allow screening of all glycosyltransferase genes simultaneously. cDNAs are typically used in printing microarrays. While not a fully proven alternative, oligonucleotides offer cost and time savings, and allow differentiating expression of highly homologous genes. Accordingly two microarrays have been evaluated consisting of a subset of glycosyltransferases probes printed on poly-lysine coated glass slides. One contains oligonucleotides (75 mer) and the other cDNA fragments (500-1200 bp) probes. Housekeeping genes have also been arrayed as positive controls and to be employed for normalization of the signal. The validity of both tools has been evaluated by examining differences in gene expression of a group of murine glycosyltransferases in resting and activated CD8+ T cells [1]. Our ultimate goal is to construct a "glyco-chip" containing all human and murine glycosyltransferases genes that have been sequenced and are available in public databases.

Reference

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C22.16

Cytokine induced expression of fucosyltransferases (FucTs) in human hepatocytes

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The family of fucosyltransferases (FucTs) show a complex tissue- and cell-type specific expression pattern. Their members display similar but differing enzymatic properties, making it possible to discriminate between the various enzymes by assessing their substrate specificity patterns. Many tissues and cells, however, contain multiple FucTs, thus creating a pattern that is a composite of the activities of the various FucTs that are expressed. To begin to unravel the mechanism by which the strictly coordinated FucT expression is regulated, we determined first which FucTs are expressed by normal human hepatocytes. The acceptor specificity of enzyme activity in cell lysates toward a diagnostic panel of substrates demonstrated that the FucT activity in hepatocytes stems from a combination of the activity of FucT VI and FucT III. Subsequently, we investigated whether factors known to induce changes in glycosylation on acute phase proteins secreted by the liver had any effect on the expression of either FucT. Thus, hepatocytes were cultured in the presence of various cytokines and changes in FucT expression were investigated by assessing enzyme activity and comparing this to the enzyme activity of non-stimulated cells.

C22.17

Polymorphonuclear granulocytes from an individual carrying an inactivating missense mutation in *FUT7* roll on E- and P-selectin

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Fucosyltransferase VII (Fuc-TVII) is involved in the final step of sialyl Lewis x (SLe^x) biosynthesis in myeloid cells. This epitope mediates binding to selectins during the recruitment of polymorphonuclear granulocytes (PMN) to activated endothelium. An inactivating missense mutation (G329A; $Arg^{110} \rightarrow$ Gln) in the Fuc-TVII coding gene *FUT7* was recently described. Here the consequences of this mutation were studied in an individual carrying the G329A-mutation homozygously. The expression of SLe^x on her PMN was studied with flow cytometry and Western blot and found to be lowered compared to non-mutated controls, whereas expression of the VIM-2 epitope was elevated. To assess the role of the G329A-mutation in P- and E-selectin mediated rolling of PMN, isolated cells were perfused over a surface coated with different selectins in an *in vitro* flow chamber system. There was no increase in rolling velocity of PMN with the G329A-mutation compared to controls on E- or P-selectin coated surfaces.

C22.18

Proposed role for CFTR in Golgi sorting of glycosyltransferases

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CFTR is the gene which when mutated causes cystic fibrosis (CF). The gene encodes a transmembrane glycoprotein with Cl^- channel function. CF has a glycophenotype which has been described in glycoproteins, glycolipids, and mucins [1]. When CF airway epithelial cells were transfected with wtCFTR and the Cl^- channel functionally corrected, the CF glycosylation pattern of the membrane glycoproteins was that of non-CF airway cells [2]. After wtCFTR was no longer detected the CF phenotype returned. The CF phenotype is expressed as increased Fuc in α1,3 linkage to GlcNAc (Fucα1,3GlcNAc), decreased Fuc in α1,2 linkage to Gal and decreased NeuAc. In the case of Fucα1,2Gal, the biosynthetic machinery in the form of enzyme activity and mRNA was present to the same extent in both CF and non-CF airway cells. The reciprocal relationship of Fucα1,3GlcNAc with Fucα1,2Gal or NeuAc in CF suggests that α1,3 FucT acts first as the glycoproteins are processing through the Golgi when CFTR is mutated. The prior action of this transferase would preclude the action of FucTII or NeuAcT. Using several models for protein sorting and transport through the Golgi we propose that CFTR contributes to Golgi sorting by virtue of its Cl^- channel function. *Supported CFF SCANLI00G0; SCANLI00Z0.*

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C22.19

Growth-dependent and branch-specific β1,3-galactosylation of Epo N-glycans in recombinant BHK-21 cells

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In our research programme aiming at the identification of the glycosylation capabilities of mammalian cell lines for the production of human therapeutic glycoproteins, we have isolated a BHK-21 cell clone expressing recombinant human Epo with novel N-glycosylation characteristics. In routine cultures of confluently grown cells, the Epo produced contained proximally fucosylated di-, tri- and tetraantennary oligosaccharides in a ratio of 3 : 4 : 7, without *N*-acetyllactosamine-repeats, but containing one β1,3-linked Gal in 50% of all structures. Interestingly, when analyzing Epo from supernatants derived from subconfluent cells, less than 15% of total oligosaccharides were found to contain the type I motif. In in vitro assays using cell lysates and complex-type oligosaccharide acceptors, β1,3-Gal-transferase activity was detected only in confluently, but not in subconfluently grown cells, assuming that the dramatic switch from type II to type I structures is mediated by upregulation of an endogenous β1,3-Gal-T(s) after establishing cell-cell contacts. The enzyme exhibited a high specificity for GlcNAc β1,4-linked to Man, since type I structures were found only in tetraantennary and 2,4-branched triantennary structures, and not in 2,6-branched triantennary or in diantennary chains.

C22.20

Delineation of the minimal catalytic domain of human ST3Gal I

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The CMP-Neu5Ac : Galβ1-3GalNAc α2,3-sialyltransferase (ST3Gal I) is a Golgi membrane bound type II glycoprotein that catalyses the transfer of sialic acid residues to Galβ1-3GalNAc disaccharide structures found on O-glycans and glycolipids. In order to gain further insights into the structure/function of this sialyltransferase, we studied protein expression, N-glycan processing and enzymatic activity upon transient expression in the COS-7 cell line of various constructs deleted in their stem region. The expressed soluble polypeptides were detected within the cell and in the cell culture media using a specific hST3Gal I monoclonal antibody. Soluble form of the protein consisting of amino acids 26-340 (hST3-Δ25) and 57-340 (hST3-Δ56) were efficiently secreted and active and their kinetic parameters were determined. In contrast, further deletion of the stem region leading to hST3-Δ76 and hST3-Δ105 gave rise also to various polypeptides that were not active within the transfected cells and not secreted in the cell culture media. In addition, the present study demonstrate that the recombinant hST3Gal I polypeptides transiently expressed in COS-7 cells are glycosylated with complex and high mannose type glycans on each of the five potential N-glycosylation sites.

C22.21

A dominant negative mutant of β1,4-*N*-acetylglucosaminyl-transferase III

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The β1,4-*N*-acetylglucosaminyltransferase III (GnT-III) plays a regulatory role in the biosynthesis of N-glycans, and it has been suggested that its product, a bisecting GlcNAc, is involved in a variety of biological events as well as in regulating the biosynthesis of the oligosaccharides. We found that GnT-III contains a small motif that is conserved in snail β1,4GlcNAc transferase and β1,4Gal transferase by comparing their amino acid sequences. Introduction of mutations into this domain demonstrated that two conserved Asp residues (Asp-321 and Asp-323 of rat GnT-III) are essential for enzymatic activity. The overexpression of Asp-323-substituted mutant GnT-III in Huh-6 cells led to the suppression of the activity of endogenous GnT-III, but no significant decrease in its expression. Structural analysis of the total N-glycans revealed that the formation of bisected sugar chains is specifically inhibited in the Asp-323 GnT-III-transfected cells. These findings indicate that the mutant serves a dominant-negative effect on the N-glycan biosynthesis. This type of "dominant negative glycosyltransferase", first identified herein has potential value as a powerful tool for defining the precise biological roles of the bisecting GlcNAc structure.

C22.22

A large family of β3Gal-Ts, β3Gn-Ts and β3GalNAc-Ts

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To date, four members of the human β3Gn-T, five members of the human β3Gal-T and one member of β3GalNAc-T have been identified. All of them share amino acid motifs in three regions of the catalytic domain. We have cloned all members and expressed them in a baculo-expression system. Their substrate specificities were determined using a series of acceptor substrates. β3Gal-T5 showed the strongest activity for the synthesis of the Galβ1,3GlcNAc linkage. β3Gn-T2 was determined to be an extension enzyme of polylactosamine chain by the strongest activity for the synthesis of the GlcNAcβ1,3Gal linkage. A new member, named β3Gn-T5, showed preferential activity toward glycolipids, resulting in the synthesis of lactotriosylceramide (Lc_3Cer). β3Gn-T5 was identified to be Lc_3Cer-synthase. The detailed specificities of all members will be presented, and their *in vivo* function for glycoconjugate synthesis will be discussed.

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C22.23

Regulation of polysialic acid by ST8SiaII and ST8SiaIV

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Polysialic acid (PSA) is an oncofetal antigen consisting of α-2,8-linked sialic acid residues attached to the neural cell adhesion molecule NCAM. Two enzymes, the polysialyltransferases ST8SiaII and ST8SiaIV, are often expressed simultaneously but are independently able to synthesize PSA. This study was undertaken to elucidate in which way the individual enzymes contribute to PSA expression. Using a semi-quantitative RT-PCR strategy PSA-positive human tumor cell lines were screened for expression of ST8SiaII and ST8SiaIV at different mRNA levels. Divergent patterns observed in some cell lines suggest that polysialyltransferases are independently regulated at the transcriptional level [1]. Moreover, ST8SiaIV mRNA expression was specifically induced by retinoic acid-treatment of SH-SY5Y neuroblastoma cells [2]. Subsequent analyses revealed that only ST8SiaIV but not ST8SiaII mRNA levels were correlated with the degree of PSA expression and the cellular capacity to synthesize PSA. Our data therefore indicate that ST8SiaIV is the major regulator of NCAM polysialylation in vivo and enables an accelerated polysialylation in specific cellular contexts. (Supported by DFG grants Hi 678/1-1 and 1-2).

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C22.24

Molecular cloning of UDP-GlcNAc:lactosylceramide β1,3-*N*-acetylglucosaminyltransferase (β3Gn-T5)

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A new member of the UDP-*N*-acetylglucosamine:β-galactose β1,3-*N*-acetylglucosaminyltransferase (β3Gn-T) family having the β3Gn-T motifs was cloned from rat and human cDNA libraries and named β3Gn-T5 based on its position in a phylogenetic tree. We concluded that β3Gn-T5 is the most feasible candidate for lactotriaosylceramide (Lc_3Cer) synthase, an important enzyme which plays a key role in the synthesis of lacto- or neolacto-series carbohydrate chains on glycolipids. β3Gn-T5 exhibited strong activity to transfer GlcNAc to glycolipid substrates, such as lactosylceramide (LacCer) and neolactotetraosylceramide (nLc_4Cer; paragloboside), resulting in the synthesis of Lc_3Cer and neolactopentaosylceramide (nLc_5Cer), respectively. A marked decrease in LacCer and increase in nLc4Cer was detected in Namalwa cells stably expressing β3Gn-T5. This indicated that β3Gn-T5 exerted activity to synthesize Lc_3Cer and decrease LacCer, followed by conversion to nLc_4Cer via endogenous galactosylation. The following four findings further supported that β3Gn-T5 is Lc_3Cer synthase. (1) The β3Gn-T5 transcript levels in various cells were consistent with the activity levels of Lc_3Cer synthase in those cells. (2) The β3Gn-T5 transcript was presented in various tissues and cultured cells. (3) The β3Gn-T5 expression was up-regulated by stimulation with retinoic acid and down-regulated with 12-*O*-tetradecanoyl-phorbol-13-acetate in HL-60 cells. (4) The changes in β3Gn-T5 transcript levels during the rat brain development were determined. Points (2),(3) and (4) were consistent with the Lc_3Cer synthase activity reported previously.

C22.25

Characterization and expression of a new protein O-mannosyltransferase *POMT2* in mouse and humans

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Protein O-mannosylation is initiated at the endoplasmic reticulum by a family of conserved mannosyltransferases (*PMT*s) [1]. It was considered as a yeast specific modification, but within the last years *PMT*-homologs were identified from *Drosophila* (*rt*) and human (*POMT1*) [2]. In additon, it emerges that O-mannosyloligosaccharides might play a substantial function in higher eucaryotes [3].
We cloned a new O-mannosyltransferase (*POMT2*) from mouse and human. Northern analyses showed that *POMT2* is expressed in all tissues with high abundance in glandular tissues, especially in testis. Western analyses and immuno-histochemical staining confirmed this data. In addition, heterologous expression in yeast O-mannosylation mutants indicates that *PMT*-homologs from higher eucaryotes catalyze a yeast-type protein O-mannosyltransfer reaction.

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C22.26

Association of the splice site mutations in the *ABO* glycosyltransferase gene with the B3 and Ael phenotypes

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The typical serology characters of the B3 and Ael subgroups are weak B transferase activity with mix-field red cell agglutination pattern and trace A antigens detectable only after absorbed and eluted from red cells, respectively. Cloning and sequencing of the seven exon regions of the *ABO* glycosyltransferase genes of the Taiwanese with B3 or Ael phenotypes demonstrated wild type coding sequences in both cases; however, point mutations of G to A at –5 nucleotide of *B3*-gene exon 3 and that of *Ael*-gene exon 6, leading to the change of the conserved splice donor sequence from GTGAGT to GTGAAT in the *B3* gene and from GTAAGT to GTAAAT in the *Ael* gene, respectively, were identified. Mutation analyses demonstrated the association of the respective mutation with the B3 and Ael phenotypes, respectively, in Taiwanese, but not with normal phenotypes. Further, *B*-gene transcript with exon 3 region skipped but not the complete *B*-gene transcript was amplified by RT-PCR from the B3 RNA resource. The normal A/B transferase posesses 354 amino acid residues. The trancript with exon 3 skipped encodes a transferase with the deletion of 19 amino acid in the N-terminal portion and the retention of the majority of C-terminal portion, which carries the enzyme activity domain. Serum-transferase assay demonstrated about one sixth of activity in B3 serum when compared with those of the normal B sera.

C22.27

Purification and characterization of dI/C2/C4 β1,6-*N*-acetylglucosaminyltransferase from rat small intestine

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A rat intestinal β1,6-*N*-acetylglucosaminyltransferase forming the β1,6-branched poly-*N*-acetyllactosamine structure has been purified to apparent homogeneity through a combination of affinity chromatographies using Q-Sepharose FF, Ni^{2+}-chelating Sepharose FF, Zn^{2+}-chelating Sepharose FF, UDP-hexanolamine-agarose, and lacto-*N*-triose II-aminocellulofine. The purified enzyme showed a single protein band with an apparent molecular mass of 66 kDa on nonreducing SDS-PAGE. The purified enzyme acted on GlcNAcβ1-3'LacNAc (LacNAc: Galβ1-4GlcNAc), and synthesized GlcNAcβ1-3'(*GlcNAcβ1-6'*)-LacNAc, but did not act on LacNAcβ1-3'LacNAc. This observation suggests that the purified enzyme forms a β1,6GlcNAc branch at the peridistal Gal residue in polylactosamine structures (dIGnT6 activity). The enzyme also converted O-glycoside core 1 and core 3 analogs, Galβ1-3GalNAcα1-O-pNP (pNP: paranitrophenyl) and GlcNAcβ1-3GalNAcα1-O-pNP, into core 2 and core 4 structures, Galβ1-3-(*GlcNAcβ1-6*)GalNAcα1-O-pNP and GlcNAcβ1-3(*GlcNAcβ1-6*)-GalNAcα1-O-pNP, respectively, with a similar rate of transfer to the dIGnT6 activity. Hence this enzyme can be appropriately named dI/C2/C4 β1,6GnT.

C22.28

Conformational change on β4Gal-T1 from an inactive to active state upon substrate binding

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Lactose synthase (LS) enzyme is a 1:1 complex between the catalytic component, β-1,4-galactosyltransferse (β4Gal-T1) and the regulatory component, α -lactalbumin (LA). The crystal structures of LS in complex with various ligands, were solved at 2 Å resolution. The overall conformation of the catalytic domain of β4Gal-T1 in LS structure, although very similar to the previously published crystal structure of free β4Gal-T1 [1] (conformation-I), the region comprising residues 345 to 365, and the side chain conformation of Trp314, adopt a different conformation (conformation-II). In the present structure residues 359 to 365 are in α-helix conformation and not in a loop conformation as in the conformation I. Because of the conformational differences observed for this flexible loop, the catalytic pocket has been redefined. The structures suggest that upon substrate binding to β4Gal-T1, the region comprising residues 345 to 365 in β4Gal-T1 undergoes a large conformational change from I to II. The present crystal structures also suggest that UDP-Gal binds to β4Gal-T1 in conformation I and upon its binding first the side chain of Trp314 reorients from outside the catalytic pocket to inside, followed by a conformational change in the flexible region from conformation I to II which creates metal, sugar acceptor and LA binding sites on β4Gal-T1.

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C22.29

α-Lactalbumin stimulates glucosyltransferase activity exhibited by β4Gal-T1: Crystal structure of β4Gal-T1•LA complex with UDP-Glc

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The β-1,4-galactosyltransferase enzyme (β4Gal-T1) transfers galactose (Gal) from UDP-Gal (a) to *N*-acetylglucosamine (GlcNAc), which constitutes its galactosyltransferase (Gal-T) activity, and (b) in the presence of α-lactalbumin (LA) to Glucose (Glc), which is its lactose synthase (LS) activity. The β4Gal-T1 also transfers Glc from UDP-Glc to GlcNAc, comprising its glucosyltransferase (Glc-T) activity, albeit at an efficiency of only 0.3-0.4% of Gal-T activity. We have shown earlier that LA increases the Glc-T activity of β4Gal-T1 by almost 30-fold, which corresponds to an efficiency of 8% of Gal-T activity. In addition, LA also enhances the Glc-T activity toward various *N*-acyl substituted glucosamine acceptors. The steady state enzyme kinetic analyses suggest that the presence of LA in the Glc-T reaction reduces the true K_m for acceptor substrates by 30-fold, whereas the K_m for UDP-Glc is only reduced by about 5-fold. The crystal structures of the β4Gal-T1•LA complex with UDP-Glc•Mn^{2+} and with *N*-butanoyl-glucosamine at 2.3 and 2.2 Å resolutions, respectively, reveal the binding sites for UDP-Glc•Mn^{2+} and the acceptor on the β4Gal-T1 molecule. The details of binding of these substrates and their relevance to the Glc-T activity will be presented.

C22.30

The oligosaccharyltransferase complex from pig liver: cDNA-cloning, expression and functional characterization

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Oligosaccharyltransferases (OSTs) catalyse the *en bloc* transfer of Dol-PP-oligosaccharides on to specific asparagine residues within the triplet-sequence Asn-Xaa-Thr/Ser. The pig liver enzyme has been purified as a hetero-oligomeric protein complex consisting of ribophorin I, ribophorin II, a 48 kDa (OST48) and a 40 kDa subunit [1]. Based on overlapping sequences from independent λ-clones isolated from a porcine cDNA-library, full-length cDNAs specific for OST48, ribophorin I and ribophorin II were reconstructed whose ORFs encoded for polypeptides with molecular masses of a 48 kDa, 68.7 kDa and a 69.3 kDa, respectively. None of these subunits were found to display OST activity when over-expressed in COS1-cells alone. By contrast, a reproducible increase of activity was observed when OST48 was co-expressed together with ribophorin I, indicating that these two subunits are responsible for the catalytic activity [2]. Immunofluorescence microscopic studies with lysine-mutants of OST48 and chimaeric proteins containing distinct domains of OST48 and Man_9-mannosidase showed that OST48 is retained in the ER by interaction with ribophorin I rather than by the dilysine retrieval signal located within its C-terminal cytosolic domain [3].

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C22.31

Analysis of the three mannosyltransferase genes required for the early assembly of lipid-linked oligosaccharide in mammals

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For the early assembly of lipid-linked oligosaccharide (LLO) on the cytosolic side of the rough ER membrane, five GDP-mannosedependent mannosyltransferases (MT-I to V) are considered to be required.
In order to characterize these mammalian MTs, we are analyzing the human and mouse genes for three enzymes, MT-I, MT-III and MT-IV. First, as for MT-I, the human gene *Hmat-1* was cloned as the ortholog of the yeast *ALG1* gene [1]. Also, the mouse gene *Mmat-1* has been recently cloned and its characterization is now in progress. In addition, human and mouse genes encoding MT-III, designated *Hmat-3* and *Mmat-3* respectively, have been successfully cloned as the ortholog of the yeast *ALG2* gene (manuscript in preparation). Finally, the expression cloning of the gene which complements the lesion of the mouse G258 mutant, defective in MT-IV, is in progress. Until now, we have identified the YAC clone 923f5, which corrects the mutation in G258, with the aid of the 1.3kb human marker DNA [2]. Thus, the current effort is focusing to the construction of BAC contig map of 923f5 to obtain the BAC clone which rescues the G258 mutation.

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C22.32

Promoter analysis of human β-1,4-galactosyltransferase (β-1,4-GalT) V gene

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Our previous study demonstrated that β-1,4-GalT V whose cDNA is cloned from human breast cancer cells can preferentially galactosylate the GlcNAcβ1→6Man group formed by *N*-acetylglucosaminyltransferase V (GlcNAcT V), and its gene expression level increases upon malignant transformation of cells. Furthermore, the gene expression levels of β-1,4-GalT V but not other members of a β-1,4-GalT family are strongly correlated with those of GlcNAcT V in human cancer cell lines. To elucidate a regulatory mechanism of β-1,4-GalT V gene expression in cancer cells, the genomic clones containing 5'-untranslated region of β-1,4-GalT V gene were isolated from a human placenta lambda genomic library. The sequence analysis of the 5'-flanking region of β-1,4-GalT V revealed that the β-1,4-GalT V gene promoter contains several putative binding sites for transcription factors including AP-1, c-Myb and an Ets family, the latter of which has been shown to be involved in the regulation of the GlcNAcT V gene in cancer cells, suggesting that the gene expression of β-1,4-GalT V in cancer cells could be also regulated by the factors involved in malignant transformation. The promoter activities in the β-1,4-GalT V gene will be discussed.

Reference

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C22.33

Genomic cloning and promoter analysis of the mouse GlcAT-P involved in the biosynthesis of the HNK-1 carbohydrate epitope

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The HNK-1 carbohydrate epitope is expressed on various cell adhesion molecules in the nevous system and is suggested to play an important role for cell-cell and/or cell-substratum interactions. The expression of the HNK-1 epitope is spatially and temporally regulated during development. Recently we isolated a glucuronyltransferase, GlcAT-P, from rat brain which is presumably the regulatory enzyme for the biosynthesis of the HNK-1 epitope. To elucidate the biological function of the HNK-1 and the molecular mechanisms regulating its expression, we cloned and characterized the mouse GlcAT-P gene and its promoter. The coding region of GlcAT-P is composed of 5 exons and 4 introns spanning 6 kb. The GlcAT-P gene is a single copy gene and maps to A4 region of mouse chromosome 9. Primer extension analysis revealed a major transcription start site (+1) at 553 bp upstream of the translation initiation codon. In transient transfection assay, the region between nucleotides –207 and +347 acted a strong promoter in PC-12 cells, but not in COS-1 cells. These results suggest that this region is involved in the specific promoter activity for the HNK-1 carbohydrate expressing cells such as PC-12.

C22.34

Characterization of two different glucuronyltransferases involved in the biosynthesis of the HNK-1 carbohydrate epitope

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The HNK-1 carbohydrate epitope is mainly expressed on many cell adhesion molecules and on some glycolipids in the nervous system, and is spatially and temporally regulated during the development of the nervous system. The HNK-1 epitope is suggested to be involved in cell-cell and/or cell-substratum interactions. Recently, we cloned two different glucuronyltransferases, GlcAT-P and GlcAT-S, which are associated with the HNK-1 epitope biosynthesis. To elucidate the reason why two different enzymes are involved in the HNK-1 epitope biosynthesis, we investigated the distribution of GlcATs in the nervous system. *In situ* hybridization analysis revealed that distribution of GlcAT-P was different from that of GlcAT-S in some brain regions. In the cerebellum, GlcAT-P mRNA was specifically expressed in the Purkinje cell layer while GlcAT-S mRNA was mainly localized in the granular layer. We also compared the acceptor specificity of these two enzymes. The GlcAT-P transferred GlcA to bi-, tri-, tetra-antennary complex type sugar chains with almost equal efficiency while the GlcAT-S transferred GlcA to these sugar chains with different efficiency. These results suggest that GlcAT-P and GlcAT-S are associated with the HNK-1 epitope biosynthesis in different brain regions and with different acceptor specificity.

C22.35

Stage-specific expression of fucosyloligosaccharides in zebrafish embryo

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Developmental control of cell surface oligosaccharides is attained by controlled expression of activities of key glycosyltransferase. Recently, two α1-3fucosyltransferase genes of zebrafish (*Danio rerio*), *zFT1* and *zFT2*, expressed during embryonic periods were cloned and their products were found to be capable of transferring fucose by an α1-3 linkage to an acceptor. Sugar chains expressed in a stage-specific manner were detected taking the advantage of high sensitivity and separation of pyridylamino sugar chains by comparing the HPLC profiles of PA-sugar chains obtained from several developmental stages. PA-N- and O-linked sugar chains from embryos were separated by DEAE-HPLC, and the neutral fraction and the acidic fraction were collected. The neutral fraction was further separated by size-fractionation HPLC and by reversed-phase HPLC. To detect a fucosyloligosaccharide(s), PA-sugar chains were analyzed by reversed-phase HPLC before and after digestion with α1-3/4 fucosidase. Two sugar chains were detected and their structures were analyzed by combining two dimensional HPLC mapping and exoglycosidase digestion.

Reference

Kageyama N *et al* (1999) *J Biochem* **125**:838-845

C22.36

Human glycosaminoglycan glucuronyltransferase I gene and a related processed pseudogene: genomic structure, chromosomal mapping, and characterization

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In this report we describe the characterization of the human glycosaminoglycan glucuronyltransferase I gene (*GlcAT-I*) and a related pseudogene. The *GlcAT-I* gene was localized to human chromosome 11q12-q13 by *in situ* hybridization of metaphase chromosomes. *GlcAT-I* spanned 7 kb of human genomic DNA and was divided into five exons. Northern blot analysis revealed a single transcript of 1.5 kb. Reverse transcription polymerase chain reaction analysis showed that *GlcAT-I* gene exhibited ubiquitous but markedly differential expression in the 18 adult and 8 fetal human tissues examined. The *GlcAT-I* promoter was approximately 3-fold more active in a melanoma cell line than in a hepatoma cell line, thus providing evidence for the differential regulation of the gene expression. Screening of a human genomic library identified one additional distinct genomic clone containing an approximately 1.4 kb sequence region which shared an overall 95.3% nucleotide identity with exons 1 through 5 of the *GlcAT-I* gene. However, a lack of intron sequences, as well as the presence of several nucleotide mutations, insertions, and deletions that disrupted the potential *GlcAT-I* reading frame, suggested that the clone contained a processed pseudogene. The pseudogene was localized to chromosome 3. Hence, the human genome contains two related *GlcAT-I* genes that are located on separate chromosomes.

C22.37

The *Drosophila* ortholog of *EXTL3* encodes α1,4-*N*-acetylglucosaminyltransferase involved in heparan sulfate biosynthesis

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Hereditary multiple exostoses gene (EXT) family members, EXT1, EXT2 and EXTL2, are glycosyltransferases required for heparan sulfate (HS) biosynthesis and their homologues in *D. melanogaster*, ttv, and *C. elegans*, rib-2, are also known to function in HS biosynthesis. Recently, we found a new *Drosophila* gene highly homologous to EXT family members, especially EXTL3, and tested its glycosyltransferase activities using various oligosaccharides as acceptors. The truncated soluble form of this protein transferred *N*-acetylglucosamine (GlcNAc) through α1,4-linkage not only to *N*-acetylheparosan oligosaccharides which represent growing HS chains but also to GlcAβ1-3Galβ1-*O*-C_2H_4NHCbz, a synthetic substrate for α-GlcNAc transferase I that determines and initiates HS synthesis. The present finding suggests that this enzyme is most likely involved in HS chain initiation and elongation in the fly and that EXTL3 probably plays the similar role in the biosynthesis of HS in human.

Reference

Sugahara K *et al* (2000) *Curr Opin Struct Biol* **10**:518-527

C22.38

Enzyme specificity of plant members of the α1,3/4 fucosyltransferase gene family

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Three members of the α1,3/4-fucosyltransferase gene family can be identified in the genome of *Arabidopsis thaliana*. cDNA clones of two of the putative *A. thaliana* fucosyltransferase genes have been expressed in Chinese hamster ovary (CHO) lec8 cells and were identified as core α1,3-fucosyltransferases involved in N-glycosylation. Transfected cells stain with a core α3-fucose specific antibody and show core α1,3-fucosyltransferase activity in an *in vitro* assay. A homolog of the third gene was isolated from a sugar beet cDNA library. COS-7 cells transiently transfected with this cDNA showed fucosyltransferase activity towards Galβ1,3GlcNAc containing structures, forming the Lewis^a (Galβ1,3[Fucα1,4]GlcNAc) epitope. Less than 1% activity was found using Galβ1,4GlcNAc or LNnT as acceptor substrate. The enzyme thus can be defined as an α1,4-fucosyltransferase and differs from mammalian members of the α3/4 fucosyltransferase family by its specificity for Lewis^a synthesis. In plants, Lewis^a containing N-glycans are found whereas typical mammalian Galβ1,4GlcNAc and Galβ1,4[Fucα1,3]GlcNAc (Lewis^x) structures are lacking. The enzyme encoded by the cloned cDNA therefore is expected to be involved in Lewis^a formation in plants.

C22.39

ST6Gal I cleavage occurs at different sites in various cell types

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ST6Gal I that adds Sia residue to the N-linked glycan chains of glycoproteins is localized in the trans Golgi and trans Golgi network in rat liver. After localizing to the Golgi, a part of ST6Gal I is cleaved and secreted. Previously we have determined that the major ST6Gal I cleavage occurred between Lys 40 and Glu 41. Here we used a synthetic peptide, Glu41-Phe42-Gln43-Met44-Pro45-Lys46-Cys, as an immunogen to preprare a cleavage site-specific antibody. The obtained antibody, αE41, successfully recognized a soluble ST6Gal I in the medium of ST6Gal I expressing cells. Next, we studied the cleavage and secretion of ST6Gal I in various cell types, and found that the proteloytic cleavage site differs from cell to cell, suggesting that cell type specific secretases act on ST6Gal I cleavage-secretion.

Reference

Kitazume-Kawaguchi S *et al* (1999) *Glycobiology* **9**:1397-1406

C22.40

Structure-function relationship of UDP-GalNAc: polypeptide *N*-acetylgalactosaminyltransferase 1 (GalNAc-T1)

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We identified two Cys residues, C^{212} and C^{214}, essential for interaction with UDP-GalNAc. GalNAc-T1 was inactivated by treatment with a Cys-specific reagent, *p*-chloromercuriphenyl sulphonic acid (PCMPS). However, the activity was retained when UDP-GalNAc was added along with PCMPS, indicating that GalNAc-T1 contains free Cys residues interacting with UDP-GalNAc. Site-directed mutagenesis of some of the conserved Cys residues resulted in complete inactivation (C106A, C330A, C339A, and C410A) or drastic decrease in the activity (C212A and C214A). Among these Cys residues, only C^{212} and C^{214}, which locate at the C-terminal to the DxH motif, were identified as free by Cys-specific fluorescent labeling of GalNAc-T1. Three Ser mutants (C212S, C214S, and C212S/C214S) exhibited higher activities than those of the respective Ala mutants, indicating the interaction of GalNAc-T1 with UDP-GalNAc through a hydrogen bond at these sites. Involvement of the introduced Ser residues in catalysis was demonstrated by the inhibition of all Ser mutants with diisopropyl fluorophosphate (DFP), while the wild type was insensitive to DFP. By contrast, Ser mutants became more resistant to PCMPS. Especially, C212S/C214S were completely resistant to PCMPS. The kinetic study of the Ser mutants also showed interaction of C^{212} and C^{214} with the sugar donor, in which the affinity with UDP-GalNAc decreased, while that with the acceptor was unaffected as compared with those of the wild type.

C22.41

A novel human Gal-3-*O*-sulfotransferase: Molecular cloning, characterization and its implications in biosynthesis of 3'sulfo-Lewis^X

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Based on sequence homology with the previously cloned human cerebroside sulfotransferase (CST) cDNA, a novel sulfotransferase was cloned by screening a human fetal brain cDNA library. The novel sulfotransferase gene was present on human chromosome 11q13. The isolated cDNA contained an open reading frame that encoded a predicted protein of 431 amino acid. The amino acid sequence showed 33 % identity with that of human CST and 38 % with that of recently cloned human β-Gal 3'-sulfotransferase, GP3ST. The recombinant enzyme expressed in CHO cells catalyzed transfer of sulfate to position 3 of non-reducing β-galactosyl residues in Galβ1-4GlcNAc. Type 2 chains served as good acceptors, while type 1 chains served as poor acceptors. Northern blotting analysis showed that the sulfotransferase mRNA was strongly expressed in the thyroid. Co-transfection of the enzyme cDNA and fucosyltransferase III into COS-7 cells resulted in expression of 3'sulfo-Lewis^X and a small amount of 3'sulfo-Lewis^a. These results indicated that the newly cloned enzyme is a novel Gal-3-*O*-sulfotransferase and is involved in biosynthesis of the 3'sulfo-Lewis^X structure.

C22.42

Observing glycosyltransferases in living cells: A green and red fluorescent protein study

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To study the intracellular distribution and function of glycosyltransferases, we fused the enhanced green fluorescent protein (EGFP) to their carboxy terminal ends and examined the behavior of the resulting fusion proteins in living cells. Using concomittant intracellular EGFP staining and cell surface labelling with rhodamine-conjugated antibodies or lectins we found that tagging glycosyltransferases with EGFP has no detectable effect on their Golgi sub-compartmentation or their *in vivo* activities. Furthermore we tested the effectiveness of using the newly cloned red fluorescent protein from the coral Discosoma (DsRed) in combination with EGFP to simultaneously examine the overlapping of glycosyltransferases within the Golgi apparatus. We found that EGFP and DsRed-conjugated glycosyltransferases behave diffenrently with respect to their intracellular distribution and the time of their appearance in the Golgi. EGFP-tagged proteins appear in the Golgi within less than 3 hours post-transfection whereas the DsRed-fused enzymes mature slowly (5-9 h) and undergo a dotted distribution, which only partly, if not at all, superimpose with EGFP-stained glycosyltransferases. Despite this fact, all DsRed-fused glycosyltransferases tested were found to be fully active in vivo. Many potential cell biological applications would flow from these studies including imaging of glycosyltransferase targeting, transport, turnover and compartmentation-dependent function. In addition, the possibility of tagging glycosyltransferases with fluorescent proteins without altering their catalytic activities in vivo, makes it suitable for protein-protein interaction sudies using the fluorescence resonance energy transfer (FRET) technology.

C22.43

Macular corneal dystrophy is caused by mutations in a gene encoding corneal GlcNAc 6-*O*-sulfotransferase which is involved in sulfation of keratan sulfate

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Macular corneal dystrophy (MCD) is an autosomal recessive hereditary disease leading to severe visual impairment. MCD is classified into two subtypes, type I and type II, as defined by either the absence or presence of sulfated keratan sulfate (KS) in the patient's serum. The gene responsible for MCD has been mapped on chromosome 16q22 by linkage analyses. We identified a novel carbohydrate sulfotransferase gene (*CHST6*) within the critical region of MCD. *CHST6* encodes a novel sulfotransferase designated as corneal *N*-acetylglucosamine 6-*O*-sulfotransferase (CGn6ST) which is involved in sulfation of keratan sulfate. In MCD type I, several mutations were identified within the coding region of *CHST6*, and these mutations lead to an inactivation in the enzymatic activity of CGn6ST. In MCD type II, large deletions and/or replacements were found in the upstream region of this gene. *In situ* hybridization analysis indicated no *CHST6* transcripts in corneal epithelial cells in a MCD type II patient. Thus, the mutations in type II lead to loss of cornea-specific expression of *CHST6*. These results explain the biochemical difference between MCD type I and type II.

Reference

Akama TO *et al* (2000) *Nat Genet* **26:**237-241

C22.44

Expression of mouse α(1,3)fucosyltransferases and sialyl-Lewis X on vascular endothelium during acute allograft cardiac rejection

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Selectin-selectin ligands interactions have been described to control leukocyt-endothelium interactions during inflammatory response. To study a particular role for α(1,3)fucosyltransferase VII (Fuc-TVII) or IV (Fuc-TIV) in L-selectin ligand biosynthesis during experimentally-induced acute allograft cardiac rejection, we transplanted hearts from Fuc-TVII -/-, Fuc-TIV-VII -/- or Fuc-TVII-ICAM-1 -/- mice into incompatible CBA/J or C3H recipients. Tissue of rejecting allografts was analyzed by immunohistochemistry with 2F3, HECA-452, 2H5 and Ca19-9 antibodies for the expression of sLeX or sLeA on the activated endothelium. Our data indicate that a defect in Fuc-TVII and Fuc-TIV gene results in a significant decrease of endothelial staining with all antibodies. Expression of Fuc-TVII and Fuc-TIV, but not Fuc-TIX, in the course of allograft rejection was also documented by RT-PCR. Graft survival data show normal rejection course in the case of Fuc-TVII -/- as well as Fuc-TIV-VII double -/- heart donors. Our data indicate that defects in the expression of Fuc-TVII and Fuc-TIV and sLeX-like carbohydrate structures in allograft endothelium do not significantly affect allograft survival and suggest a role for another leukocyte-endothelial interactions.

C22.45

Molecular cloning and expression of a novel *N*-acetylgalactosamine-4-*O*-sulfotransferase

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N-Linked oligosaccharides terminating with the sequence SO_4-4-GalNAcβ1,4GlcNAcβ1,2Manα are present on the pituitary hormone lutropin (LH), thyrotropin, and proopiomelanocortin. The sulfated structures on LH are essential for expression of its biologic function *in vivo*. Here we report the cloning and characterization of a novel *N*-acetylgalactosamine-4-*O*-sulfotransferase designated GalNAc-4-ST2 which is a type II membrane protein and displays a high degree of amino acid sequence identity with GalNAc-4-ST1 [1] and other members of the HNK-1 sulfotransferase family. GalNAc-4-ST2 [2] transfers sulfate to the C-4 hydroxyl of terminal β1,4-linked GalNAc in the sequence GalNAc-β1,4-GlcNAc-R found on N-linked oligosaccharides and non-terminal β1,4-linked GalNAc in chondroitin and dermatan. GalNAc-4-ST2 is most highly expressed in trachea. The cloning and characterization of a second GalNAc-4-sulfotransferase, GalNAc-4-ST2, that is closely related to GalNAc-4-ST1 but clearly has distinct properties adds further strength to the view that the sulfated saccharides produced have critical biological roles.

References

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[2] Kang H-G *et al* (2001) *J Biol Chem* **276:**10861-10869

C22.46

Mouse kidney tubular epithelial cell-specific regulation of glycochains and megalin

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β6GlcNAc transferase (core 2 GNT) is regulated in a proximal tubular cell-specific manner in mouse kidney. Compared with mutant strains of inbred mice which lack this core 2 GNT mRNA expression, wild strains express a large amount of the enzyme and modify glyco-chains of particular glycolipids and glycoproteins. To identify what glycoproteins are modified by the core 2 GNT *in vivo*, we detected molecules with antibody against core 2-Le^x structure. A purified molecule is large molecular weighted protein (~600k) by SDS-PAGE and binds to lentil lectin Sepharose. The molecule is immunoprecipitated by anti-megalin antibody. Thus we conclude that the membrane glycoprotein modified by the core 2 GNT is megalin whose function is reabsorption of low molecular weight proteins from glomelular filtrate. The possibility of functional modulations of megalin by proximal tubule-specific glyco-chains, including affinity to ligands, stability, intracellular trafficking is the subject of further research.

Reference

Sekine M *et al* (2001) *Eur J Biochem* **268:**1129-1135

C23. Glycotechnology

C23.1

Total stable labeled carbohydrates synthesized by methylotrophic bacteria

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It was found that obligate methylotroph *Methylobacillus flagellatum* KT produces exopolysaccharides which consist of fructose only, and pink pigmented facultative methylotroph *Methylobacterium extorquens* excretes significant amount of exopolysaccharides composed of glucose and altrose. These properties are persisted on media total labeled by stable isotopes (^{2}H, ^{13}C). Chemical composition of isolated polysaccharides were investigated by TLC and HPLC after hydrolysis with sulfuric acid. The level of fructose deuteration was determined by structural analysis pentaacetate ^{2}H-labeled fructose by heteronuclear NMR spectroscopy.
Strains have been selected with increased synthesis of polysaccharides on total ^{2}H-, ^{13}C-labeled media. It is possible to obtain gram-scale amount of [U-^{2}H]-fructose in laboratory fermenter. This method is suitable for biosynthesis of multi isotope labeled carbohydrates also.

C23.2

Large scale production of Lewis^{X} analogues by metabolically engineered *Escherichia coli*

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We have recently described a new fermentation process [1] that makes possible the high yield production of lacto-*N*-neotetraose (LNnT) by recombinant strains of *Escherichia coli* overexpressing *lgtA* and *lgtB* genes from *Neisseria meningitidis* (encoding β-1,3-*N*-acetylglucosaminyltransferase and β-1,4-galactosaminyltransferase, respectively).
We show now that this process can be extended to the synthesis of fucosylated derivatives of LNnT by coexpressing the *lgtAB* genes with the α-1,3-fucosyltransferase, *futB* gene, from *Helicobacter pylori* [2] in a metabolically engineered *E. coli* strain that overexpresses the genes of GDP-fucose biosynthesis which take part of the colanic acid gene cluster.
Lacto-*N*-neofucopentaose, Gal-β(1→4)-GlcNAc-β(1→3)-Gal-β(1→ 4)-[Fuc-α(1→3)]Glc, was identified as the main product (3g/L) but a significant amount of lacto-*N*-neodifucohexaose containing the Le^{x} epitope was also obtained.

References

[1] Samain E (2001) Patent FR2796082
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C23.3

Glycosaminoglycan-transfer reaction using endo-β-xylosidase as a glycotechnological tool for artificial synthesis of neoproteoglycan

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Transglycosylation reaction of glycosidase is regarded as a reverse reaction of hydrolysis, and useful in glycotechnology. Previously, we have isolated and characterized endo-β-xylosidase from mid-gut gland of the mollucs *Patinopecten* [1]. This enzyme is an endo-type glycosidase which hydrolyzes Xyl-Ser linkage between glycosaminoglycan (GAG) chain and core protein of proteoglycan (PG), and as a result, releases intact GAG chain from PG. In this study, we investigated transglycosylation activity of endo-β-xylosidase, as a model for the transferation of GAG chain into peptide. Peptidoglycan from bovine tracheal cartilage as a donor and *p*-nitrophenylglycerol (PNPG) as an acceptor were incubated with endo-β-xylosidase, and reaction mixture was subjected to HPLC analysis. As a result, a reaction product was observed after the reaction. Then, the reaction product was analyzed by enzyme digestion, cellulose acetate membrane electrophoresis, and ion-spray mass spectrometry. The results indicated that the reaction product was the glycosaminoglycated PNPG with a chondroitin sulfate chain (Mr=30k). It was confirmed that the chondroitin sulfate chain of the donor was transferred to the acceptor PNPG by the transglycosylation reaction of endo-β-xylosidase. It is highly likely that application of the transglycosylation reaction using endo-β-xylosidase would facilitate artificial synthesis of proteoglycan having new physiological functions.

Reference

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C23.4

Biological activity of therapeutic glycoproteins is influenced by glycosylation

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Most of the therapeutic glycoproteins produced by the pharmaceutical industry are recombinant molecules. In the last few years attention has been drawn to the importance of the carbohydrate part of these substances. Different methods of production can result in different carbohydrate structures and these latter changes can affect the biological properties. Recombinant plasminogen (Pg) and humanized monoclonal antibodies (IgG) were used as models to further examine the effect of carbohydrate structure on biological properties. In the case of Pg, carbohydrate mutants were prepared that lacked various glycosylation sites, whereas IgG glycosylation was altered when it was produced in different host cells and under different culture conditions. The glycosylation profiles of the different forms were assessed using a lectin/ELISA, a lectin/surface plasmon resonance method and monosaccharide content. The lectins used were AAA, ConA, DSA, MAA, PNA, and SNA and the monosaccharide content was measured by HPAEC/PAD after acid hydrolysis. Biological properties of Pg and IgG were assessed by cleavage/activity assays and immunoassays respectively. The Pg mutants exhibited reduced biological activity. This suggests that manipulation of carbohydrate structure may be useful for enhancing therapeutic properties.

C23.5

Host-dependent glycosylation of a recombinant glycoprotein

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Genetic modification of organisms to produce recombinant proteins is already an established technique. Many recombinant proteins are already available as therapeutics (e.g. insulin) and for industrial uses (e.g. enzymes). They can be produced in a range of organisms from bacterial or yeast cells to plants or animals. Although such techniques enable the expression of foreign proteins in a host organism, the glycosylation of that protein is under the control of the host. This can be a particular problem when producing therapeutic glycoproteins since the glycans might have an impact on the structure as well as on the function and half-life of the glycoprotein in the body. In this investigation a human protein (hX) has been expressed in the milk of mice (mrX) and rabbits (rrX) and the N-linked glycans of these recombinant proteins were compared to those of the human counterpart from plasma. It was shown that the N-linked glycosylation of the protein varies when it is expressed in different organisms, although all the major structures identified were diantennary and varied only in degree of fucosylation and sialylation. It was also concluded that the expression of transgenic glycoproteins in different hosts provides an opportunity to investigate the differences in the glycosylation machinery of different organisms.

C23.6

Controlling N-glycosylation of recombinant glycoproteins produced in plants

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Plants have potential as safe factories for the production of recombinant proteins. Several heterologous proteins like antibodies have successfully been produced in plants. Mammalian glycoproteins produced in plants will, however, have a plant like N-glycosylation. Glycosylation can have a dramatic effect on the characteristics and functionality of glycoproteins. Our aim is to control plant glycosylation by both knocking out plant glycosyltransferases and introducing mammalian enzymes in plants. We have succesfully introduced human β1,4galactosyltransferase [1] and other mammalian glycosyltransferases in tobacco plants. The N-linked glycosylation in these plants is altered and now has both plant and mammalian characteristics. Surprisingly, the plants have a normal appearance. Our results show that mammalian enzymes are active in plants and their introduction has no dramatic effect on the plant physiology.

Reference

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C23.7

Comparison of the glycosylation pattern of a Mab type IgG2b purified from different culture conditions

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The cell line hybridoma CB.Hep-1 secretes a Mab type IgG2b, specific for a determinant of rHBsAg [1,2]. This Mab is used as an immuno ligand in the downstream purification process of rHBsAg, employed for a commercially available recombinant hepatitis B virus vaccine (Heber-Biovac HBTM, Heber Biotec S.A., Cuba). Cells were cultivated in 250 ml stirrer spinner-flasks, a hollow-fiber bioreactor (HFB), T-25 flasks, and a miniPERM system. Protein-free medium (TurboDoma HP-1 / 500 μM ferric citrate), low-protein-content medium (HyQSFX-Mab / 1% FCS), and serum-containing medium were used for each system, and harvesting was carried out under saturation conditions. N-Glycosylation profiling was performed after labeling of released N-glycans with two different fluorophore tags, 4-aminobenzoic acid (4ABA) and 8-amino-1,3,6-naphthalene trisulfonic acid (ANTS). 4ABA derivatives were analyzed by NH_2-HPLC, and ANTS derivatives using a combination of FACE and NH_2-HPLC. 4ABA derivatives showed the highest resolution and sensitivity, being more adequate for Mab IgG glycan characterization.

References

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C24. Glycotherapeutics

C24.1

Structure-dependent inhibition of the selectin-mediated cell adhesion by partial synthetic glucan sulfates

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The leukocytes recruitment into tissues during inflammation is a multistep process requiring their adhesion to the endothelium. The initial adhesive event is the selectin-mediated tethering and rolling of leukocytes along the vessel wall. Inhibition of this early step in inflammation may be an interesting strategy for anti-inflammatory therapy. Besides sialyl-Lewis x derivatives, sulfated polysaccharides are known to interfere with this process. We developed a new class of partial synthetic glucan sulfates (GS) with significant *in vivo* anti-inflammatory activity. The aim of the presented study was to establish structure-activity relationships of their inhibitory influence on the selectin-mediated cell adhesion. In adhesion assays, the GS inhibit the L- and P-, but not the E-selectin-mediated cell adhesion. Their activity depends not only on the degree of sulfation and the molecular weight but also on the sulfation pattern. Further, the basic polysaccharide structure was shown to play an important role, e.g. the GS are considerably more active than heparin. These results obtained under static conditions correlate well with the effects observed in a dynamic test system. The latter examines the influence of the test compounds on the interactions of selectin expressing cells with a vascular surface imitate containing sialyl-Lewis x under shear flow. In conclusion, the cell adhesion inhibitory potency of GS can be regulated by structural modifications of GS and is suggested to contribute to the overall *in vivo* activity of these promising new anti-inflammatory compounds.

C24.2

Production of human α-galactosidase in *Saccharomyces cerevisiae* for replacement therapy of Fabry disease

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Fabry disease is an X-linked inborn error of glycolipid metabolism caused by deficiency of the lysosomal α-galactosidase. In order to produce the more economic therapeutics for replacement therapy of Fabry disease, we introduced human α-galactosidase gene into *S. cerevisiae* mutant that disrupted the outer chains of mannan and expressed. The recombinant α-galactosidase had not only neutral [$Man_8GlcNAc_2$] type but also acidic [$(Man\text{-}P)_{1\text{-}2}Man_8GlcNAc_2$] type sugar chains. Because mannose-6-phosphate (Man-6-P) residue is needed to incorporate the α-galactosidase into the lysosome of the cells, we trimmed down the sugar chains of the enzyme by a bacterial α-mannosidase. The α-galactosidase treated with the α-mannosidase had a Man-6-P residue on a non-reduced end of oligosaccharide chains. Uptake of the α-galactosidase by the fibroblasts of the Fabry patient was three times higher than non-treated α-galactosidase, and the uptake was inhibited by 5 mM Man-6-P. Incorporated α-galactosidase was targeted to the lysosome and degraded ceramide trihexoside in the fibroblast of the Fabry cells for 6 days.

C24.3

Expression of oligosaccharide-pp-dolichol is essential for angiogenesis

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Angiogenesis *in vivo* is a complex, multi-step process and occurs in stages that orchestrate a network of cooperative interactions. These include (i) initiation, (ii) progression, (iii) differentiation, and (iv) stabilization and maturation. Earlier studies have suggested that N-linked glycoproteins are important determinants of endothelial cell proliferation. Using a capillary endothelial cell line as a model which expresses the blood clotting antigen Factor VIIIC, a 270 kDa N-linked glycoprotein, we have investigated the importance of oligosaccharide-pp-dolichol (OSL) in the angiogenic process. Upon exposure to tunicamycin (TM), OSL expression as well as DPMS activity were reduced. As a consequence, surface blebbing, cell shrinkage, compaction of nuclei showing pyknotic appearance, loss of membrane contact with neighboring cells, and membrane fragmentation occurred. This led to G1 arrest and induction of apoptosis. Cellular adhesion to collagen IV > poly-L-lysine > collagen I > fibronectin > laminin was also altered to poly-L-lysine > collagen IV > collagen I ≥ fibronectin > laminin with an increase in $[Ca^{2+}]_i$ in TM treated cells. TM action was time and concentration dependent, observed in serum as well as FGF-2 stimulated cells, was reversible but could not be mimicked by cycloheximide. TM however, had no effect when added either in mid-G1 or in mid-S. On the other hand, 8Br-cAMP protected the cells from the apoptotic action of TM. The release of ER stress, however, was not mediated by the MAP kinase pathway but by enhancing the DPMS activity by PKA-dependent phosphorylation.

C24.4

Gal ls S1 - in vivo and in vitro evaluation of the anti-angiogenic and anti-inflammatory activity

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Based on possible anti-angiogenic and anti-inflammatory activities sulfated carbohydrates are interesting leads for the development as chemopreventive agents. Therefore the sulfated α-1,4-galactan Gal ls S1 (DS = 1.29, MW = 13.5 kD) was tested in vitro and in the CAM-assay for its anti-angiogenic, anti-inflammatory and anti-tumor activity. Modifications of the CAM-assay are suitable in vivo models to study the embryonal as well as the pathological angiogenesis. Side effects can be detected in the same system. To find out possible modes of action, the interaction with VEGF and FGF-2, important regulators of angiogenesis, was studied in vitro by determination of VEGF in supernatant of U-87 MG cells, in the FGF-2 trypsin digestion assay and in fluorospectrometric analysis. At a concentration of 50 µg/egg Gal ls S1 exhibited a good anti-angiogenic (score 1.14) and anti-inflammatory effect (inhibition 69%) but only an uncertain effect in glioma-induced angiogenesis. Results obtained in the in vitro assays revealed that Gal ls S1 interacted with FGF-2 but was not able to inhibit the VEGF production in cell culture. The results demonstrate that Gal ls S1 is a potent candidate for a further drug development as chemopreventive agent.

Reference

Paper DH *et al* (1995) *Macromol Symp* **99**:219-225

C24.5

Inhibition of tumor growth by a new glycosaminoglycan isolated from the African giant snail *Achatina fulica*

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Acharan sulfate is a new type of glycosaminoglycan from the giant African snail *Achatina fulica.* This polysaccharide has a primarily repeating disaccharide structure of α-D-*N*-acetylglucosaminyl 2-sulfo-iduronic acid. In the previous report, acharan sulfate inhibited FGF-2 mitogenicity in the presence of heparin [1]. We have examined the effects of acharan sulfate on the inhibition of angiogenesis not only by chorioallantoic membrane assay, but also proliferation of bovine capillary endothelial cells. Systemic subcutaneous administration (10 mg and 30 mg/kg) of acharan sulfate significantly inhibited the growth of murine Lewis lung carcinoma implanted intramuscularly in syngeneic C57BL/6 mice without toxicity or resistance. It is suggested that the antitumor activities of acharan sulfate might be due to the inhibition of angiogenesis. (Supported by the BK21 project).

Reference

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C24.6

A dual action by honey bee honey on alkaline phosphatase

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Nothing as yet has been studied about honey bee honey biochemical effects, its action on alkaline phosphatase was studied because of the enzyme biological importance of constituting part of biological membranes. Honey diluted 1:10 was added in vitro to the partially purified liver enzyme from mice. At different concentrations of substrate honey brought about enzyme activation with a lower Km followed by inhibition. Kinetically honey produced enzyme-substrate complex with a longer pre-steady state. Mg^{2+} increased alkaline phosphatase activity and Zn^{2+} ions reversed this effect. Honey antagonises partially Zn^{2+} inhibitory effect that a prior full occupation of Zn^{2+} at the enzyme M_1, M_2, M_3 sites is a must for Mg^{2+} activation. Previously, Awad indicated a certain ratio between Zn : enzyme and/or Mg : enzyme to get full enzyme activity while honey by its suggested generation of new enzyme conformation, polymerization <-> depolymerization action thus reducing the enzyme metallospecificity as supposed to affect -vely or +vely the above ratio then an apparent dual action of honey on the enzyme activity an increased activation energy and a discontinuity in the Arrhenuis plot may be understood. The presence of honey resembling Zn^{2+} action leads to at low substrate concentration occupation of the first high-affinity type and at high substrate concentration the second low-affinity type of enzyme site becomes occupied. Honey contains high concentration of phosphate preventing enzyme dephosphorylation of Ser-102 producing inhibition and that the octahedral ligand environment of magnesium is replaced by a distorted tetrahedral environment about the zinc.

Reference

Awad OM ElDin *et al* (1975) Enzymes **20**:221-33

C26. Lectins

C26.1

Galectin-8 functions as a novel matricellular protein

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Galectin-8 is a secreted mammalian lectin, having two carbohydrate recognition domains (CRDs) joined by a link peptide. We have recently shown that galectin-8 modulates cell adhesion by forming protein-sugar complexes with cell surface integrins. In the present study we could demonstrate that immobilized galectin-8 was equipotent to fibronectin (Fn) in promoting cell adhesion and spreading in serum-free medium. Truncation of the C-terminal CRD of galectin-8 largely abolished its ability to support cell adhesion, indicating that both CRDs are required to maintain a functional adhesive galectin-8. Cell adhesion to galectin-8 was mediated by sugar-protein interactions with integrins; accordingly, it was potentiated in the presence of Mn^{2+}, while it was interrupted in the presence of soluble galectin-8, integrin b_1 inhibitory antibodies, EDTA, or thiodigalactoside, but not by RGD peptides. Adhesion to galectin-8 triggered integrin-mediated signaling cascades such as Tyr phosphorylation of FAK, Paxillin and $P130^{cas}$, followed by activation of a Rho-family GTPases, MAPK and PI3K cascades. This was associated with a different cytoskeletal rearrangement when compared to cells adherent to Fn. In contrast, soluble galectin-8, like soluble Fn, inhibited cell adhesion. Additionally, soluble galectin-8 was found to bind serum Fn, thus, it could inhibit cell adhesion upon interaction with integrins either alone or as a complex with Fn. Taken together, these findings implicate galectin-8 as a novel matricellular protein. When immobilized, it promotes cell adhesion trough integrins. In contrast, when present as a soluble ligand, it interacts both with integrins and other matrix proteins to negatively regulate cell-matrix interactions.

C26.2

Novel sialic acid binding lectins from different parts of *Saraca indica*: purification and molecular characterization

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Two lectins, one from the seed integument of *Saraca indica* and another from the filament of its flower have been purified to homogeneity - the former one by a combination of affinity chromatography on porcine thyroglobulin-Sepharose 4B, Sephadex G-50 and Protein PAC 300 SW in HPLC, the later one successively by Sephadex G-200 and Resource Q in FPLC and affinity chromatography on fetuin-Sepharose 4B. Both lectins are monomeric with MW ~12kD and 65kD, respectively, by SDS-PAGE, and agglutinated human A, B and O and animal erythrocytes; the agglutination titer of neuraminidase treated erythrocytes of all kinds was found to be diminished. The lectins possess two sugar binding sites as revealed by fluorescence quenching studies in presence of Neu5Ac. Circular dichroism studies performed on both lectins with and without carbohydrate ligand give a hint to their conformations. The detailed carbohydrate-binding specificities of the lectins determined by enzyme-conjugated lectin inhibition assay have shown that saracin is highly specific for binding Neu5Ac whereas filament lectin exhibits its highest specificity towards Neu5Ac-α2,6Galβ1,4GlcNAc. Saracin induces apoptosis in activated human T cells, thus seems to be an immunomodulator for the mammalian immune system.

C26.3

Isolation of a trypsin inhibitor from *Peltophorum dubium* seeds with lectin-like activity

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Lectins and proteinase inhibitors are frequently found in Leguminosae seeds. Proteinase inhibitors are classified in several families, including Kunitz-type inhibitors which are Mr 20000 proteins. They are useful tools in the study of different biochemical processes, such as platelet aggregation, blood coagulation, fibrinolysis and inflammation. The initial aim of this work was to isolate a lectin. An extract of *Peltophorum dubium* seeds was submitted to affinity chromatography on a tyroglobulin-agarose column. The fraction with hemagglutinating activity on rabbit erythrocytes was further purified on a Superdex-75 column. Two bands were detected on SDS-PAGE, corresponding to 20 and 22 kDa, both in presence or absence of reducing agent. Nevertheless, only one peak was obtained by HPLC on a C4 column. The amino-terminal sequence of both bands was DFVLDVEGIFLR NGGIYYILPD... This sequence has 73% identity with Kunitz-type soybean trypsin inhibitor. Therefore, trypsin inhibitory activity of the isolated protein was determined using BAEE as substrate and the Ki obtained was 1.6 x 10^{-7} M. Hemagglutinating activity was Ca^{2+} dependent and hemagglutination inhibition assays showed that neuraminic acid, tyroglobulin, colominic acid, triganglioside GT_{1b} and heparin were potent inhibitors. These results encourage future studies about the effects of this protein on different biological systems, in view of its dual lectin and protease inhibitor properties.

C26.4

Molecular cloning and characterization of a novel collectin CL-K1

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Collectins are a family of proteins that contain at least two characteristic structures, a collagen-like region and a carbohydrate recognition domain. There are four groups of collectins; the MBP group, the SP-A group, the SP-D group, and the fourth newly isolated CL-L1. Here we report that the molecular cloning of a novel collectin from kidney (CL-K1) which belongs to the fourth family of collectin. CL-K1 has a unique character that it can produce major two spliced variants. *CL-K1* gene has seven exons including another starting exon (exon 1b) between the exon 1 and 2 of *CL-L1* gene having six exons. CL-K1 having the exon 1a is a secreted protein and CL-K1 having the exon 1b is a non-sectered type protein since it has signal peptide or not. RT-PCR study shows that secreted CL-K1 is expressed ubiquitously and non-secreted CL-K1 is expressed only in kidney and testis. Transfection of expression vector encoding Myc-His tag fragments indicates that both genes can be translated in proteins which one is secreted and another exsists in nuclear and cytoplasm. The sugar specificities of CL-K1 from transfected cells are mannose, fucose, and N-acetylgalactosamine but their affinities are low. A study on the functions in these fourth member collectins is in progress.

Reference

Ohtani K *et al* (1999) *J Biol Chem* **274:**13681-13689

C26.5

Localization of annexin and endogenous ligands in bone and cartilage

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Annexins are calcium-dependent phospholipid-binding proteins widely distributed in lower to higher eukaryotes. Annexin V is a matrix vesicle-enriched protein that binds to type-II and type-X collagen. We found previously that annexin V recognizes acidic glycoconjugates. Since calcified and extracellular bone matrix contain various acidic glycoconjugates, they may be endogenous ligands of annexin. To test this hypothesis, we examined localization of annexin V and glyco-conjugates in bone and cartilage.
Immunohistochemical analysis with antibodies and lectins, showed that annexin V, osteopontin and type-X collagen were localized intra-cellularly in chondrocytes and periosteum of 13-day chick embryo tibia. Chondroitin sulfate localized in the extracellular matrix. Biotinyl-*Macchia amurensis* mitogen, a lectin that recognizes terminal sialic acid and/or sulfate on carbohydrate chains, stained chondrocytes intracellularly and periosteum, and biotinyl-annexin V stained perio-steum. Therefore, we can suggest that the endogenous ligands of annexin V are sialylated and/or sulfated glycoproteins of the periosteum.

C26.6

Interaction of a lectin from the mussel *Crenomytilus grayanus* with polyvalent neoglycoconjugates

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A Gal/GalNAc specific lectin from the sea mussel *Crenomytilus grayanus* (CGL) exhibits a high affinity to mucin-like molecules [1]. But the mechanism of this interaction is still unknown. We studied CGL binding to polyacrylamide conjugates and liposomes with various sugar epitopic density as simple models of natural ligands. We observed a positive cooperativity of CGL for binding to β-Gal residues on polyacrylamide. Binding constants increased from 134 to 600 mM^{-1} at increasing sugar density from 10 to 40% galactose density. We also carried out experiments with α- and β-GalNAc derivatives. Constants for the β-form were higher as compared with the α-GalNAc residues. For specification of the mechanism of the lectin interaction with the cell surface we investigated the CGL interaction with glycoliposomes prepared from neoglycolipid (4-(cholesterylsuccinoylamino)-phenyl-β-D-galactopyranoside). The positive cooperative effect of binding was observed at Gal densities from 1.5% up to 14%, and a negative effect was detected at densities higher than 14%. The constants for glycoliposomes with 2.5-14.3% of neoglycolipid was 3.5- 7.2 nM^{-1}. In comparison with the neoglycopolymers investigated, the CGL binding constants to glycoliposomes increased 10-100 times.

Reference

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C26.7

Expression of galectin-3 in PC12 cells involves Ras/MAPK-dependent signaling pathway

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Galectin-3 (gal-3) is a member of the galectin family of lectins whose expression strongly depends on the cell state, i.e. its expression can be induced during cell activation or transformation [1,2]. Here we show that in PC12 cells the expression of gal-3 protein is regulated via the activation of Ras- and mitogen-activated protein kinase (MAPK) dependent signaling pathways but independent of neuronal differentiation: (i) Gal-3 expression and neurite outgrowth are reversibly induced by nerve growth factor (NGF) and basic fibroblast growth factor, but not by ciliary neurotrophic factor, epidermal growth factor, insulin or interleukin-6. (ii) In NGF-treated PC12 cells, gal-3 expression and neurite outgrowth are inhibited on the level of TrkA by K252a, on the level of Ras by N-acetylcysteine, and on the level of MAPK by PD 098059 and U0126. (iii) In the PCMTras21 PC12 subclone, zinc-inducable expression of an oncogenic form of Ras leads to expression of gal-3 and neurite outgrowth. These data demonstrate a significant contribution of Ras-/MAPK-dependent signaling pathways to the expression of gal-3 in PC12 cells.

References

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[2] Walther M *et al* (2000) *J Neurosci Res* **61:**430-435

C26.8

Molecular cloning and characterization of a novel collectin CL-P1

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Collectins are a C type lectin family which have collagen-like sequences and carbohydrate recognition domains (CRD). They are involved in host defense since they can bind to carbohydrate antigens of microorganisms and inhibit their infection by direct neutralization and agglutination, and opsonization by collectin receptors. Here we report a new scavenger receptor of the membrane type collectin from placenta (collectin placenta 1=CL-P1). The cDNA has an insert of about 2.2 kb coding for a protein containing 742 amino acid residues. It resembles type A scavenger receptor since the scavenger receptor cysteine rich domain (SR-CR) is substituted by a CRD. Northern analyses, RT-PCR and immunohistochemistry show that CL-P1 is expressed in mainly in vascular endothelial cells but not in macrophages. Expression studies show that CL-P1 can bind and phagocytose not only bacteria (*Escherichia coli* and *Staphylococcus sureus*) but also yeast (*Saccharomyces cerevisiae*). Furthermore it reacts with oxidized low density lipoprotein (OxLDL) but not acetylated LDL. These results indicate that CL-P1 plays important roles in host defenses that are different from those of soluble collectins, including the regulation of regenerated LDL.

Reference

Gough PJ and Gordon S (2000) *Microbes and Infection* **2:**305-311

C26.9

The epitope of the H-Type 2 trisaccharide recognized by *Erythrina corallodendron* lectin. The first observation of both strong hydrophobic and attractive polar interactions for complex formation involving a lectin

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Various deoxy- and mono-*O*-methyl derivatives of the H-Type 2 human blood group determinant (Fucα2Galβ4GlcNAc) and of LacNAcβOMe were employed in an enzyme linked lectin assay to examine the specificity requirements of *Erythrina corallodendron* lectin (ECorL) and the contribution of the fucose moiety to the binding of the above trisaccharide to the lectin. Based on the relative activities of the ligands, and the X-ray structure of the ECorL/LacNAc complex, the epitope of the trisaccharide recognized by ECorL has been delineated. The fucose moiety is shown to provide a strong hydrophobic interaction at its 2-OH, most likely with Trp 135 of the lectin combining site, in addition to the polar interactions of the key hydroxyl groups at positions 3 and 4 of the galactose and the stacking of the latter residue on Phe 131. Comparison of the epitopes of the trisaccharide recognized by three other legume lectins, from *Galactia tenuiflora*, *Psophocarpus tetragonolobus* and *Ulex europaeus*, shows that all four are different, in spite of the high similarity between their tertiary structures. Our findings provide a striking example of how a single oligosaccharide can carry different messages for communication with different recognition molecules.

* Deceased July 22, 2000.

C26.10

Structure of a fungal galectin, *Agrocybe cylindracea* lectin

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Agrocybe cylindracea lectin has a unique carbohydrate-binding specificity, recognizing NeuAcα2,3Galβ1,3/1,4GlcNAc-/1,3GalNAc sequences. By the analyses of peptide fragments and cDNA, we determined the complete primary structure of total 161 residues. This lectin has a CRD of galectin including 7 amino acid residues commonly found in galectins. *Agrocybe* lectin has 36% sequence identity with fungal galectin 1 from *Coprinus cinereus* fruit bodies. Analysis with TOF-MS suggested that initial Met was deleted and the next Ser was acetylated. In comparison with *Coprinus* galectin 1, residues 43-47, 91, 103, 104, 131, 132, 142, 143 and 153 were inserted, and 4 residues corresponding to residues 51-54 of human galectin 1 were deleted. The crystal structure of *Agrocybe* lectin was analyzed at 2.0 Å. The space group is $P2_12_12_1$ with a = 57.07, b=96.31 and c = 121.66 Å. Final Rcryst was 19.5% and Rfree was 25.4%. Like animal galectins, it is composed of stranded β-structures. However, the loop structures are different from those of animal galectins, especially loops 34-47, 54-58, 75-84 and 93-103.

References

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C26.11

ZG16p, an animal protein homologous to jacalin-related plant lectins, binds to O-linked Galβ1-3GalNAc/GalNAc structures

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ZG16p is a 16 kDa protein found in pancreatic acinar cells and gut goblet cells in rat. In pancreatic acinar cells ZG16p is localized in the zymogen granules, and its expression levels respond to hormonal stimulation, suggesting that the protein is involved in regulating the secretory pathway in these cells. However, the molecular mechanisms of the biological functions of ZG16p are still unclear [1]. ZG16p shows a remarkable similarity to a Moracea plant lectin, jacalin; therefore, we supposed that ZG16p possesses lectin activities important for exhibition of *in vivo* functions. When we examined binding activities of rat ZG16p to glycoproteins by affinity chromatography, human immunoglobulin A and bovine submaxillary mucin, both of which have O-linked Galβ1-3GalNAc and/or GalNAc sugar chains, were found to be good ligands for ZG16p. On the contrary, no binding to bovine RNase B, which has N-linked high mannose-type sugar chains, was observed. RT-PCR analysis of human tissues showed that ZG16p is expressed in the colon, pancreas, liver and kidney at various levels. Possible roles for ZG16p as receptors of O-linked glycoproteins in extracellular spaces or in the process of sorting and transportation through the intracellular secretory pathway are implicated.

Reference

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C26.12

High level expression of human galectin-8-thioredoxin-fusion-protein in *E. coli*

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Galectins are defined by binding to β-galactosides and conserved secuence elements. To date the family contains 10 known members, a number that probably will increase with the identification and characterization of new genes. Galectins are participants in many basic functions but their overall biological role is still obscure. Efficient expression of recombinant galectin is a requirement for evaluation of its functions in biological assays. Here we present a high level expression of thioredoxin-fused galectins for further use in functional studies. cDNA of galectin-8 was amplified using PCR with special designed primers. The PCR-product was allowed to anneal with pET-32 Ek/LIC vector, resulting in a construct where the galectin-8 is fused with thioredoxin. This construct was transformed into the *E. coli* protein expression host, BL21 Star (DE3). Overnight cultures were pelleted, bacteria lysed and the cleared supernatant was tested for galectin binding activity using fluorescence polarization. If activity was detected the cleared supernatant was allowed to pass a lactosyl-Sepharose column. Active thioredoxin-fused galectin bound to the column was eluted with a 150 mM lactose-buffer. This high level expression stategy is also applied to the separate carbohydrate binding domains of galectin-8 as well as to other galectins.

Reference

Leffler H (2001) *Results Probl Cell Differ* **33**:57-83

C26.13

Solubility-insolubility transition of a self-aggregating lectin, sophoragrin, is caused by a signal oligosaccharide

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Mannose/glucose-specific sophoragrin, a *Sophora japonica* bark lectin, shows unique self-aggregation and dissociation accompanied by transitions between insolubility and solubility, which are modulated by both a specific sugar and Ca^{2+} concentration. Sophoragrin is composed of four subunits, a-1, a-2, b-1, and b-2, and all except for b-2 are reported to be glycosylated [1]. In this study, we found and identified the specific oligosaccharide that is responsible for the self-aggregation of sophoragrin.

Biotinylated sophoragrin bound sugar-specifically to subunit 'b' but not to subunit 'a' on the membrane. Sophoragrin was labeled by only one of the photoaffinity-probes [2] derived from sophoragrin-glycans, and the labeling was inhibited by a specific sugar. In contrast, another probe-mixture, which contains major oligosaccharide of sophoragrin ($Man_3Xyl_1Fuc_1GlcNAc_2$), did not label sophoragrin. These results reveal that the self-aggregation of sophoragrin is caused by a particular signal oligosaccharide on subunit 'b'. The structure of the signal oligosaccharide will be presented. It was shown that sophoragrin can form a soluble complex with other endogenous glycoproteins, and may play a specific role through its transition between soluble and insoluble forms.

References

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C26.14

The rat brain lectin that recognized the moieties of both high-mannose oligosaccharide and peptide of glycoprotein

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Almost lectins are known to associate with only carbohydrate moieties of glycoconjugates. Accordingly, mannose recognition lectins in animals were usually detected by affinity gels coupled with mannan, high-mannose oligosaccharides or mannose containing neoglycoproteins. Recently, it is reported that a few animal lectins associate with both carbohydrate and protein moieties. Therefore, we attempted to detect mannose recognition lectin, which bound to both carbohydrate and peptide moieties of glycoconjugates, with affinity gels coupled with various glycoproteins possessing high-mannose oligosaccharides. We found that mannose recognition lectin, which bound to RNaseB, was detected in rat brain. We analyzed that this lectin was identical to glucosidase II judging from N-terminal sequence and molecular weight analysis. Furthermore, we studied this lectin-RNaseB binding modality. In the binding analysis glucosidase II to RNaseB, we found that glucosidase II recognized with both carbohydrate and peptide moieties of RNaseB. Now, we are determinating the strict binding specificity and elucidating its relationship to known function of glucosidase II.

C26.15

In vitro analysis of anti-tumor activity of mannan-binding protein

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Serum mannan-binding protein (MBP), a Ca^{2+}-dependent mammalian lectin specific for mannose, *N*-acetylglucosamine and fucose, activates complement through the lectin pathway. In addition, MBP has an anti-tumor activity in animal experiments, which appears to be independent of complement activation. We propose calling this activity MBP-dependent cell-mediated cytotoxicity (MDCC). In order to elucidate the mechanisms of the MDCC reaction, we have established an *in vitro* assay system. Using this system, it was shown that soluble cytotoxic factors were released into medium from monocytic cells upon binding to tumor cells (a human colorectal carcinoma cell line, SW1116) in the presence of MBP. MBP-ligand sugar chains isolated from SW1116 cells were indicated to initiate the MDCC reaction. In addition, we found that ligand-bound MBP stimulates polymorphonuclear leukocytes (PMN) to induce cell aggregation and superoxide production. The biological response of PMN to ligand-bound MBP was inhibited by pertusis toxin.

Reference

Ma Y *et al (1999) Proc Natl Acad Sci USA* **96**:371-375

C26.16

Characterization of oligosaccharide ligands expressed on SW1116 cells recognized by mannan-binding protein

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Mannan-binding protein (MBP), a Ca^{2+}-dependent mammalian lectin specific for mannose, *N*-acetylglucosamine and fucose, is an important serum component associated with innate immunity. We have found that MBP is cytotoxic to a human colorectal carcinoma *in vivo* through a mechanism that is called MBP-dependent cell-mediated cytotoxicity (MDCC) [1]. In order to elucidate the mechanism of MDCC, we analyzed the oligosaccharide ligands expressed on SW 1116 cells which MBP recognizes and binds to. FITC-MBP binding to SW1116 cells was inhibited by fucose-specific lectins and by mAbs against Lewis-type antigens. The cell surface glycoproteins carrying MBP ligands were isolated from the surface-labeled cells and analyzed by glycosidase digestion. Pronase-glycopeptides containing MBP ligand oligosaccharides from SW1116 cells which had been metabolically labeled with ^{3}H-glucosamine were isolated on a MBP column. The oligosaccharides were prepared by hydrazinolysis of MBP-binding glycopeptides, then pyri-dylaminated and analyzed for their monosaccharide compositions and reducing-end sugars. MBP ligands were found to be high molecular size oligosaccharides with high fucose content.

Reference

[1] Ma Y *et al* (1999*) Proc Natl Acad Sci USA* **96**:371-375

C26.17

The difference of sugar-binding specificities between Siglec-7 and -9

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Siglec-7 (sialic acid-binding Ig-like lectin-7) and -9 are members of the Siglec-3(CD33)-related subfamily. Both of them contain an N-terminal V-set domain and two C2 set domains. Furthermore, the amino acid sequence of Siglec-7 is 84% identical to that of Siglec-9, suggesting that they have recently arisen by gene duplication. In the present study, Siglec-7 or -9 was stably expressed on CHO cells by cDNA transfection to investigate their sugar-binding specificities. The transfected cells were treated with sialidase prior to binding assays, in which we used multivalent glyco-probes carrying various kinds of oligosaccharide ligands. Siglec-9 bound to sialylα2,3galactose as well as sialylα2,6-galactose. On the contrary, Siglec-7 bound to these structures poorly, but bound to other unique sialyl-oligosaccharides well, indicating that sugar-binding specificity of Siglec-7 is quite different from that of Siglec-9 despite high degree of sequence identity between them. We have already prepared various chimeric molecules between Siglec-7 and -9. We will perform the binding assay using these chimeric molecules to identify the amino acid sequence(s) that is responsible for the difference in sugar-binding specificity.

C26.18

Measurement of the human serum mannan binding proteins by FELISAC using pseudopolysaccharides and antibodies against C4/C4b

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FELISAC (functional ELISA of complement) was developed to study carbohydrate binding serum proteins. For this purpose, soluble model polyacrylamide (PAA) glycoconjugates such as (Manα-)$_{oligo}$-PAA, (GlcNAcβ-)$_{oligo}$-PAA and (Gal)$_{oligo}$-PAA as well as yeast mannan were immobilized in wells of the modified polystyrene microplate ("Medpolimer", Moscow). Immobilized (Manα-)$_{oligo}$ or (GlcNAcβ-)$_{oligo}$ were specifically interacted with a number of plant lectins as well as serum mannan binding lectin purified using (yeast mannan)-Sepharose or (Manα-)$_{oligo}$-glass beads and agarose. The yeast mannan or pseudo-mannan immobilized in microplate triggered classical complement pathway when contacting to the guinea pig complement reagent R4 and then to human serum dilutions in the presence of Ca^{2+}. Consumption of the human C4 was measured using horseradish peroxidase-labeled rabbit antibodies (fraction IgG) against human C4/C4b. The data were supported by the hemolysis functional assay of the serum C4. The possible involvement of the serum natural antibodies is discussed.

C27. Molecular glycobiology

C27.1

Characterization of GalCer expression factor-1 (GEF-1) functional domains in MDCK cells

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We previously reported that GEF-1 induced GalCer expression and morphological changes in COS-7 cells. In this study, we describe the characterization of GEF-1 in MDCK cells. Overexpression of GEF-1 in MDCK (MDCK/GEF-1) cells showed sulfatide expression as well as morphological changes. The enzyme activity and the mRNA level of UDP-galactose: ceramide galactosyl-transferase (CGT) increased in MDCK/GEF-1 cells compared with control cells. GEF-1 molecule is composed of a zinc-finger (Z), a proline-rich (P), a coiled-coil (C), and a proline/glutamine-rich (Q) domain. MDCK cells transfected with various GEF-1 deletion mutants were examined. The cells transfected with Z-domain deletion mutant (MDCK/PCQ) and those with both Z- and P-domains deletion mutant (MDCK/CQ) were similar to those with a wild-type GEF-1 (MDCK/ZPCQ) in shape, exhibiting fibroblast-like cells, whereas those with the other deletion mutants showed no morphological changes, exhibiting typical epithelial-like cells. On the other hand, MDCK/ZPCQ, MDCK/PCQ, MDCK/CQ, and MDCK/Q cells expressed sulfatide, whereas those with the other deletion mutants showed neither GalCer nor sulfatide expression. Thus, the correlation between fibroblast-like cells in shape and the glycolipid expression was good in these deletion mutants except MDCK/Q cells, suggesting that only the Q-domain may be essential for the role of GEF-1 in inducing CGT mRNA, whereas the Q-domain together with the C-domain may be required for the induction of morphological changes in MDCK cells.

C27.2

Molecular cloning and tissue distribution of mouse galectin-4

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Galectins, soluble-type lectins, have been found to participate in many biological processes such as immune response, development, signal transduction or metastasis. Galectin-4, a prototype molecule of monomer divalent galectin members, has been localized in alimentary tract of several mammalian species. In mouse, Northern blot data indicated specific expression of galectin-4 and/or -6 solely in the stomach, small intestine and colon [1]. In our study, we detected galectin-4 mRNA in several mouse tissues, including small intestine, liver, kidney and heart using RT-PCR. In addition, galectin-4 remains to be expressed in the transplanted mouse heart during acute allograft cardiac rejection. To confirm that galectin-4 and not galectin-6, the closest galectin-4 homologue, has been detected we cloned galectin-4 full-length cDNA from the mouse liver cDNA library using 3'-RACE and 5'-RACE approaches. Subcloning and sequencing of the three independent clones confirmed galectin-4 sequence identity. Thus, galectin-4 is indeed expressed in the liver tissue. These data indicate that this molecule is more broadly distributed and suggest that biological function(s) of galectin-4 may not be limited to the GI-tract. Cloning of full-length mouse galectin-4 cDNA allows the preparation of recombinant galectin-4 and anti-mouse galectin-4 specific antibody.

Reference

[1] Gitt *et al* (1998) *J Biol Chem* **273**:2954-2960

C27.3

Galactosylalkylacylglycerol, the precursor of seminolipid, is increased in the testis of saposin A and prosaposin-deficient mice

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Sphingolipid activator proteins (saposins (SAPs) A, B, C, D) are derived from a common precursor protein (prosaposin, proSAP), and specifically activate *in vivo* degradation of sphingolipids with short carbohydrate chains. A mouse model of proSAP deficiency closely mimics the human disease with an elevation of multiple glycolipids [1]. The recently developed SAP-A$^{-/-}$ mice showed a chronic form of globoid cell leukodystrophy, suggesting the role of SAP-A as an activator for galactosylceramidase to degrade GalCer [2]. ProSAP gene also encodes the Sertoli cell major sulfated glycoprotein and late spermatids are affected in the testis of proSAP$^{-/-}$ mice. Seminolipid (SM4g), the essential glycolipid for normal spermatogenesis [3], is synthesized by sulfation of its precursor galactosylalkylacylglycerol (GalEAG) in the early phase of spematocyte development. The testis of proSAP- and SAP-A-deficient mice showed normal SM4g levels, indicating that the leptotene-zygotene stage, when SM4g biosynthesis begins, is not affected. In contrast, GalEAG increased up to 5 times the normal level at 100 days in the testis of SAP-A$^{-/-}$ mice and ~150% at the terminal stage (34 days) of proSAP$^{-/-}$ mice, whereas it decreased with age in the wild-type mice. These results indicate that SAP- A is indispensable for *in vivo* degradation of GalEAG as well as GalCer.

References

[1] Fujita N *et al* (1996) *Hum Mol Genet* **5**:711-725.
[2] Matsuda J *et al*, submitted.
[3] Fujimoto H *et al* (2000) *J Biol Chem* **275**:22623-22626

C27.4

Molecular cloning of two *Arabidopsis thaliana* UDP-galactose transporters by complementation of a deficient Chinese hamster ovary cell line

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Nucleotide-sugar transporters form a family of structurally related transmembrane proteins that transport nucleotide-sugars from the cytoplasm to the ER and Golgi lumen. In these compartments, activated sugars are substrates for various glycosyltransferases involved in oligo- and polysaccharide biosynthesis. The *Arabidopsis thaliana* genome contains about 40 putative members of this transporter gene family, but for none of them the transport activity has been identified. By an expression cloning strategy using a Chinese hamster ovary cell line (Lec8) that is deficient in UDP-galactose transport, two *A. thaliana* UDP-galactose transporter cDNA clones have been isolated. Protein sequences translated from the isolated cDNAs are mutually only 20% identical and show very limited sequence identity to UDP-galactose and other transporters isolated from mammalian, yeast and protozoan origin. However, due to structural and topological characteristics both are clearly members of the nucleotide-sugar transporter family.

C28. Mucins: metabolism/function

C28.1

Chemical characterisation of the high molecular weight glycoconjugate fraction of human tears

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It is the purpose of this study to chemically characterise, using amino acid and monosaccharide analysis the high molecular weight glycoconjugates found in human tears. Basal tears were collected from patients of both genders with a range of ages. These tears were pooled and then purified by size exclusion chromatography on a Pharmacia Superose 12 column in 50mM phosphate buffer pH 7.2 that contained 1M NaCl and 1mM DTT. The fraction eluting at the void volume was collected. Gas phase hydrolysis in 6M HCl followed by F-Moc derivatisation was used to determine the amino acid content of the fraction, while monosaccharide composition was calculated by acid hydrolysis and separation with Dionex Carbo-Pac PA10 column with a pulsed amperometric detector. The amino acid composition of tear glycoconjugates is lower in serine and threonine but higher in Asx and Glx than is expected for mucins. Also the monosaccharide composition reflects a much higher N-linked component than would be expected for the mucins that have been describe previously. The chemical composition of high molecular weight glycoconjugates is not consistent with that of the mucins that have been reportedly found in the tear film of humans. Therefore another component not so-far described must be a major contributor to this part of the tear film.

C28.2

Development of monoclonal antibody, RGM23, recognising the gastric mucin in the surface mucosa of rat stomach

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A novel monoclonal antibody, designated RGM23, was generated against rat gastric mucin. RGM23 reacted with the purified mucin subunits, which had been attached to the ELISA well. This reaction was inhibited by the treatment of trypsin, but not by the oxidation with periodate, indicating the peptide moiety of the mucin molecule to be the epitope of RGM23. Histochemical study indicated that RGM23 was able to stain the mucous cells in ethanol fixed-paraffin embedded sections of surface mucosa of corpus and antrum in rat stomach but not gland mucosa in stomach or the mucosa of oesophagus, duodenum, small intestine or large intestine. RGM23 reacted with the mucin fractions on Sepharose CL-4B chromatography of the extract from rat stomach, and those on CsTFA equilibrium centrifugation. On Q-Sepharose chromatography, the surface mucin subunits separated into three fractions, and RGM23 reacted with two of them, one stained with PAS and the other stained with HID, but not with the third one. The results supported the hypothesis that the surface mucin in rat gastric mucosa may consist of the mucin subunits bearing two types of core proteins.

Reference

Goso Y *et al* (1999) *J Biochem* **126:**375-381

C28.3

Increased expression of MUCI, MUC5B and MUC8 genes in endometrial carcinomas and investigation of alternate splicing of MUC genes (MUC1 and MUC8) in female reproductive tissues and carcinomas

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Mucins comprise an important class of tumor-associated antigens. Recently our laboratory has reported the characterization of novel human airway mucin gene, MUC8 (Am.J. Respir. Cell Mol. Biol. 16, 232-241, 1997). The objectives of this investigation was (1) to determine and quantitate the expression of MUC8 and four other MUC genes, MUC1, MUC2, MUC5AC and MUC5B in reproductive tissues and carcinomas and, (2) to determine whether MUC1 and MUC8 genes undergo alternate-splicing in the endometerial and cervical tissues and carcinomas. The first objective was investigated using Northern and slot-blot analyses of the RNA isolated from the control and carcinomas tissues. The second objective was investigated by RT-PCR experiments using primers specific to certain regions of MUC1 and MUC8. The experiments showed that all five MUC genes are expressed in the reproductive tissues studied. Levels of expression of MUC1, MUC5B and MUC8 were considerably higher in endometerial adenocarcinomas as compared to those observed in non-cancerous endometrium. Of these three MUC genes, MUC1 level was the highest in the tumor tissues. The RT-PCR data obtained from RNA isolated from control and tumor tissues showed that all regions of MUC1 studied and certain regions of MUC8 showed no tissue-specific or tumor-specific processing of the respective genes. In contrast, RT-PCR experiments showed that amplification of the repeat regions of MUC8 gave only 8 or 9 repeats compared to 22 repeats reported earlier by us in human tracheal tissue. These data suggest tissue-specific processing of MUC8 in reproductive epithelia. Supported in part, by NIH grant HL34012.

C28.4

Expression of the recombinant C-terminus of the human MUC2 mucin and studies on its role in the formation of disulfide bond stabilized MUC2 dimers

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Due to the human MUC2 mucins large size it is hard to study by conventional methods. Therefor the cysteine-rich C-terminal domain of the human MUC2 apomucin was expressed as a fusion protein with the GFP and *myc*Tag sequences in CHO-K1 and LS174T cells. The Ig κ-chain signal sequence was used to direct the construct to the secretory pathway. Immunoprecipitations of [^{35}S]-labelled samples, from CHO-K1 cells expressing the construct, followed by SDS-PAGE analysis showed that the C-terminus formed disulfide bond stabilized dimers that were secreted into the media. The secreted forms have, in both cell lines, been shown to have a higher apparent molecular weight compared to the intracellular forms when separated by SDS-PAGE. This difference was, in the case of CHO-K1 cells, at least partly due to differences in glycosylation. The C-terminus expressed in LS 174T cells was also forming heterodimers with the full-length wild type MUC2. This argues for that the recombinant C-terminus can be used as a model for studying the role of the MUC2 C-terminus in the assembly and function of the MUC2 mucin.

C28.5

Specific alterations in rat mucin O-glycan expression upon parasitic infection – one of these caused by the induction of a blood group A transferase

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Structural characterisation - mainly using GC-MS, MS and GC - of rat small intestinal Muc2 O-glycans, collected at different stages of the infection of the intestinal parasitic nematode *Nippostrongylus brasiliensis*, revealed that three specific glycosylation alterations were induced transiently during the infection.
Among the sialylated oligosaccharides, there was a down-regulation of NeuGc on behalf of NeuAc, and an induction of the terminal epitope Sd^a/Cad (NeuAcα2-3(GalNAcβ1-4)Galβ1-). The shift in sialic acid is due to the down-regulation of CMP-NeuAc hydroxylase, shown by northern blotting.
Among the neutral oligosaccharides, there was an induction of terminal blood group A epitopes (GalNAcα1-3(Fucα1-2)Galβ1-). In a northern blot we see the up-regulation of a gene similar to the human blood group A transferase. During the attempts to clone this rat blood group A transferase, two similar but slightly different sequences have been found, suggesting that there might be two blood group A transferases in rat.

Reference

Karlsson NG *et al* (2000) *Biochem. J.* **350:**805-814

C28.6

Mucin glycosylation in mice altered by host-microbe interactions

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The major protein component of mucus is mucins, which consist of approximately 80% by weight of O-linked glycans with a high structural diversity. Mucins act as a physical barrier to intruding microbes and are thought to have an active role in the interaction with the internal microbial flora through dynamic alterations of the oligosaccharide expression. It has recently been shown that alterations in O-glycosylation occur upon intestinal infection in rats [1].
In this study our aim is to investigate how the glycosylation pattern changes in the mucins of mice during an intestinal infection. The nematode *Nippostrongylus brasiliensis* is commonly used in experimental systems to study inflammation in the small intestine and it was used in this study to induce the infection. The O-linked oligosaccharides of the Muc2 mucin of the small intestine of both infected and uninfected C57BL/6 mice were released. The neutral oligosaccharides were tested for their interactions with the lectins PNA and UEA 1 in an immunofluorometric assay, analyzed by monosaccharide composition analysis and structurally characterized with GC and GC-MS. Preliminary results indicate that a transient induction of two terminal oligosaccharide epitopes occurs.

Reference

[1] Karlsson NG *et al* (2000) *Biochem J* **350:**805-814

C28.7

Co-localisation of MUC5B and Sulfo-Lewis^c distinct from MUC7 and GP 340 in human salivary glands

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Saliva contains a number of heavily glycosylated proteins, e.g. high M_r mucins (MUC5B), low M_r mucins (MUC7) and salivary Agglutinin, which is identical to GP340, a member of the scavenger domain receptor family. These glycoproteins share a number of carbohydrate epitopes, including blood-group antigens. Using mAbs we have examined the localisation of MUC5B, the sulfated Lewis^c antigen, MUC7 and GP340 in two types of salivary glands: The submandibular gland, containing both serous and mucous acini, and the labial gland, containing mainly mucous acini. The localisation of the various glycoproteins was examined on formalin-fixed sections. MUC5B was present in virtually all mucous acini of both types of glands, whereas the sulfo-Lewis^c epitope was found only in a subpopulation of these mucous acini. Double labelling illustrated that in the submandibular gland the sulfo-Lewis^c epitope co-localised with MUC5B. On the contrary, MUC7 was localised in both glands in serous acini, serous cells lining the ducts, and demilune cells, capping mucous acini. GP340 localisation resembled that of MUC7, albeit less prominent in duct cells. In conclusion, MUC5B with distinct glycoforms are localised within distinct mucous acini of a particular salivary gland, while MUC7 is not at all found in mucous cells.

C29. Neuroglycobiology

C29.1

Characterization of the carbohydrate moiety of NGC, a brain-specific part-time proteoglycan with a single EGF module

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Neuroglycan C (NGC), a transmembrane chondroitin sulfate proteoglycan (CSPG), is a part-time proteoglycan (PG); a PG form in the developing cerebellum and a non-PG form in the mature one [1]. This suggests that the chondroitin sulfate (CS) moiety could be involved in the neural development. Site-directed mutagenesis revealed that NGC had only one CS side chain. We characterized both the CS moiety and other carbohydrate moieties of NGC purified from brains of 10-day-old rats, and compared them with those of neurocan and phosphacan, other brain-specific CSPGs. The disaccharide composition of the CS chain of NGC was different from those of other CSPGs; higher concentrations of 4S- and E-units, and a lower concentration of 6S-units. Lectin-blot analyses showed that NGC contained both *N*- and *O*-linked oligosaccharides. DSL, a lectin from *Datura stramonium*, intensely recognized NGC and phosphacan, but not neurocan. Con A, which recognized both phosphacan and neurocan, did not recognize NGC. The HNK-1 carbohydrate could be detectable on NGC as well as neurocan and phosphacan. These findings indicate that NGC has carbohydrate moieties structurally different from those of neurocan and phosphacan.

Reference

[1] Aono *et al* (2000) *J Biol Chem* **275:**337-342

C29.2

Localization and characterization of N-glycans from bovine brain N-CAM

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The neural cell adhesion molecule (N-CAM) was purified from brains of newborn calves. After tryptic digestion, glycopeptides containing polysialic acid (PSA) or the HNK1-epitope were isolated by serial immunoaffinity chromatography. PSA-glycopeptides were subjected to mild acid treatment and resulting products were analyzed chromatographically. PSA-fragments comprising up to 30 sialic acid units were identified confirming the polymeric nature of the PSA chains. PSA-, HNK1- and non-PSA/HNK1-glycans were enzymatically released, labelled with 2-aminopyridine and characterized by HPLC, linkage analysis, MALDI-TOF-MS and nano-ESI-MS. Edman degradation and MALDI-TOF-MS of the deglycosylated peptides revealed PSA-glycans to be exclusively linked to glycosylation sites 5 and 6, whereas glycans bearing a HNK1-epitope could be assigned to sites 2, 4, 5 and 6. Non-PSA/HNK1-glycans were found to reside at glycosylation site 2. Hence, our results allow a direct comparison of the glycosylation pattern of bovine N-CAM with N-CAM from other mammalian species.

Reference

Liedtke S *et al* (2001) *Glycobiology* **11**, in press

C29.3

Sulfatide and GM1 modulate differently the dopamine uptake in rat striatal synaptosomes: evidence for the involvement of their ionic charges

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The purpose of this study was to examine the effects of the anionic glycolipids GM1 and galactosylsulfatide on the high-affinity dopamine (DA) uptake in isolated nerve-endings from rat striatum. Synaptosomes were incubated in a Krebs-Ringer buffer with exogenous glycolipids, and then washed to remove the unbound fraction of glycolipids. After 1 h of incubation with 1.2 µM of GM1, 0.28 nmol were stably associated to synaptosomes. This association modified the activity of the neuronal dopamine transporter (DAT), since a decrease in both Vmax (13%) and Km (22%) was observed, reflecting a slight reduction of the number of uptake sites, but especially an increase of the affinity. Treatment of synaptosomes with 1.2 µM sulfatide, which possesses a double charge sulfate group, led to a higher decrease of the Vmax and Km values (19% and 36%, respectively). Sulfatide bound to synaptosomes in a greater extent than GM1 (1.0 nmol of sulfatide stably associated). Conversely, when GM1 and sulfatide were replaced by GM1 alcohol and galactosylceramide, respectively, which lack the anionic groups, no modification of the DA uptake occurred. These neutral glycolipids incorporated into the synaptosomal membranes in the same extent than the related anionic compounds.
Altogether, these data demonstrate the key role of negative charges linked to the oligosaccharide chains of glycolipids in the modulation of DA transport across the synaptosomal membrane.

C29.4

CD1 expression on glial murine cells

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CD1 gene family may present particular types of foreign Ags, such asmycobacterial lipid Ags or synthetic hydrophobic peptides. Most CD1isotypes share the unusual property of being recognized by a high frequency of naturally autoreactive alpha-beta Tcells. In the case of mouse CD1.1 a significant fraction of theautoreactive T cells express semi-invariant TCRs. CD1.1-specific T cellshave a restricted tissue distribution and verypromptly secrete a large panel of potent cytokines, including IL-4 and IFN-gamma. CD1 expression have been described in glial cell during multiplesclerosis. In the other hand, NK cells are present in MS inflammatorylesions and represent a large part ofthe lymphocytes present in the infiltrate described during EAE. We showhere, using primary cultures from WT mice and CD1 KO mice, that mouseglials cells express CD1 and are recognized by some autoreactive T cellshybridoma. All together, these results suggest that CD1 and CD1-restricted T cells may be involved in theregulation of some immune responses in the brain.

Reference

Se-Ho Park *et al* (1998) *J Immunol* **160**:3128-3134

C29.5

Importance of complex gangliosides in the maintenance of neural functions: analysis with knock-out mice of glycosyltransferase genes

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Complex gangliosides-lacking mice were born and grown up with no definite defects in the morphogenesis of nervous system. However, the mutant mice exhibited degenerative changes in the peripheral nerves, ganglia and spinal cords with aging. During the examination of the progress of neurodegenerative features of the mutant mice, clear differences in the susceptibility between male and female mutants have been observed. Generally, male mutant mice exhibited dysfunctions earlier than the wild male mice. For example, male mutants exhibited abnormal gait at 35 weeks old, while female mutants did at 50 weeks after birth. In Rota-rod test, male mutant mice did not show any progress, whereas female learned gradually. Further more, in GD3 synthase gene knock-out mice (lacking b-series gangliosides), only male mutant mice exhibited abnormal behaviors, indicating that involvement of hormonal effects in the regulatory functions of complex gangliosides is fairly great.

C29.6

Different ganglioside GD1a structures in human sensory and motor spinal roots

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Anti-ganglioside antibodies are implicated in certain autoimmune neuropathies. A subclass of Guillain-Barré syndrome, acute motor axonal neuropathy (AMAN), differentially affects motor axons while sparing sensory axons in the same nerve. AMAN is often marked by the presence of high affinity IgG antibodies against the ganglioside GD1a. The reason why the motor axons are the main target of the immune response in AMAN remains to be elucidated, especially since GD1a is a major ganglioside in both motor and sensory nerves. Purified separate ganglioside fractions from the dorsal (sensory) and ventral (motor) roots of human cauda equina were analyzed by HPTLC. Based on sialic acid staining, no quantitative difference in total GD1a expression was detected. However, a qualitative difference was seen. In certain solvent systems ventral root GD1a appeared as a double band, whereas dorsal root GD1a appeared as a single band. All bands were GD1a-related, based on binding of 4 different GD1a-specific monoclonal antibodies. TLC-blotting to PVDF membranes, combined with MALDI-TOF mass spectrometry, proved to be a sensitive method for ganglioside analysis. Whereas ganglioside GM1 species were indistinguishable between dorsal and ventral roots, distinct differences in GD1a structures were revealed. The patho-physiological significance of these differences have yet to be determined. (Supported by the National Institutes of Health and the National Multiple Sclerosis Society).

C29.7

Gangliosides in the control of nerve regeneration: Polyvalent crosslinking of nerve cell GD1a or GT1b inhibits neuritogenesis

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The major brain gangliosides GD1a and GT1b are specific ligands for the cell-cell interaction lectin, myelin associated glycoprotein (MAG). MAG inhibits neuronal regeneration and is responsible, in part, for limited axon regeneration in the central nervous system. The mechanism by which MAG binding modulates nerve cell physiology has yet to be established. Gangliosides are concentrated in small (~50 nm) dynamic membrane rafts, where they exist as polyvalent arrays. Certain signal transduction molecules are also found in membrane rafts. A possible mechanism by which MAG inhibits neuritogenesis is by stabilizing polyvalent ganglioside arrays, thereby recruiting or activating signaling molecules. We modeled this signal transduction pathway using new and highly specific IgG-class anti-ganglioside monoclonal antibodies, added with and without secondary antibodies to mimic different degrees of ganglioside crosslinking.

Without secondary antibodies, anti-GT1b or -GD1a antibodies had no effect on neuritogenesis from primary neurons in culture. When added with secondary antibodies, anti-GT1b or -GD1a remarkably inhibited neuritogenesis, a result similar to that observed in presence of MAG. Anti-GM1 antibody had no effect on neuritogenesis with or without secondary antibodies. These results support the conclusion that stabilization of GT1b or GD1a in polyvalent arrays may be the primary molecular event in its inhibition of neuritogenesis by MAG. (Supp. by NIH and National Multiple Sclerosis Society).

C30. Polysaccharides: metabolism/function

C30.1

Rheological characteristics of acetyl fucoidan isolated from commercially cultured *Cladosiphon okamuranus*

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We have isolated an acetyl fucoidan (L-fucose:D-xylose:D-glucuronic acid:acetic acid:sulfuric acid=4.0:0.03:1.0:2.0:2.0) from commercially cultured *Cladosiphon okamuranus* [1,2]. The yield of the acetyl fucoidan was estimated to be 2.0%(w/w; wet algae), while an alginate was 0.1%. The annual production of this alga in Okinawa was reported to have been approximately 15,000t/2000. The chemical structure of the acetyl fucoidan has been reported to be α-1,3-fucan and half of the L-fucose residues were sulfated at C-4 [3].The acetyl fucoidan has been prepared on an industrial scale from *Cladosiphon okamuranus* and used as an additive to health foods and drinks in Japan. We reported here the rheological characteristics of the acetyl fucoidan. The acetyl fucoidan showed shear-thinning behavior at various concentrations (1.0-2.0%) at 25°C. The viscosity and dynamic viscoelasticity increased with an increase in concentration. The dynamic viscoelasticity decreased gradually with increase in temperature. A little increase in the dynamic viscoelasticity was observed upon addition of NaCl and $CaCl_2$ (0.1%). The dynamic viscoelasticity showed a high value at a wide range of pH 3-11. The acetyl fucoidan may adopt a random conformation in aqueous solution.

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C30.2

An assay method for pectin synthase using fluorogenic oligogalacturonic acids and successive glycosyltransfer activity of the enzyme from pollen tubes in *Petunia axillaris*

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An assay for a pectin (homogalacturonan) synthase using fluorogenic oligogalacturonic acids (OGAs) as acceptor substrates was developed. The reducing end of OGA was labeled with a fluorescent reagent, 2-aminopyridine. Pyridylaminated (PA-) OGA of degree of polymerization (DP) 4 to 25 were separated by anion exchange HPLC. When a homogeneous PA-OGA of DP 14 as an acceptor and UDP-GalA as a donor were incubated with the enzyme solubilized with 0.5 % Triton X-100 from pollen tubes in *Petunia axillaris*, PA-OGAs of DP 15 to 27 were produced in 2 h, indicating that the enzyme had the activity for successive glycosyltransfer of more than 10 GalAs from UDP-GalAs to the non-reducing ends of PA-OGAs. This successive activity was time- and enzyme concentration dependent. Optimum enzyme activity was observed at pH 7.0 and 30 °C. PA-OGA of DP 13 to 15 gave maximum activity among PA-OGAs of DP 5 to 15 investigated. The pollen tube enzyme had an apparent K_m value of 6.7 μM for PA-OGA of DP 11 and a V_{max} value of 125 pmol min^{-1} mg^{-1} protein. The usefulness of fluorogenic oligosaccharides for assay of polysaccharide synthases and the characteristics, including the processive glycosyl-transfer activity, of the enzyme from *P. axillaris* pollen tubes were discussed.

C30.3

The fucoidanases in marine invertebrates

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Thirty three species of marine invertebrates were tested for fucoidan hydrolase activity using fucoidans from the brown seaweeds *Laminaria cichorioides* and *Fucus evanescens.* Fucoidanase activity was found in all species studied with molluscs, sea urchins and some arthropods possessing highest one. The crystalline style of *Spisula sachalinensis* and the hepatopancreas of *Littorina kurila* were chosen for isolation and detailed study of fucoidanases. All isolated fucoidanases catalyzed the hydrolysis of low sulfated fucoidan from *F. evanescens,* but not the highly sulfated one from *L. cichorioides.* They had optimum activity at pH 4.5-5.5, and K_m ca. 1 mg/ml. Fucoidanases of *S. sacchalinensis* produced a wide range of oligosaccharides and monosaccharides, and fucoidanases of *L. kurila* produced mainly monosaccharides. The monosaccharides were identified as non-sulfated fucose. Fucoidanases from *L. kurila* are exo-enzymes, whereas fucoidanases from *S. sacchalinensis* are endo-type enzymes.

C30.4

UDP-Glucose dehydrogenase, a key regulator in the biosynthesis of glycosaminoglycans?

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UDP-Glucose dehydrogenase (UDP-GDH) performs a two-step oxidation of UDP-glucose to UDP-glucuronic acid. The product is utilized in glycosaminoglycan biosynthesis. Due to the high sequence identity of this enzyme from bacteria and plants to mammals, we study the possible, regulatory role of UDP-GDH on polysaccharide production in those three systems. In this abstract we only report results from the bacterial system.

The *E. coli* K5 capsular polysaccharide consists of repeated disaccharide units: GlcA-GlcNAc. Possibly, the availability of UDP-GlcA is rate limiting for K5 biosynthesis. We transformed JM 109 *E. coli* with the complete gene cluster for K5-production and -export and in addition an extra plasmid with or without one more copy of UDP-GDH. Enzyme activity is 15 times higher in the overexpressor as compared with the control. K5 production is monitored by metabolic labeling with radioactive Glc and subsequent purification of produced K5. Overexpression of UDP-GDH leads to a ~3-fold decrease in formation of K5. We found no difference in length of K5 between control and overexpressing strains. The decrease in K5 amount is most probably due to fewer K5 chains. The results indicate that UDP-GDH is not rate-limiting for K5 production.

C31. Proteoglycans: metabolism/function

C31.1

Heparin oligosaccharide chain length requirements for inhibition of pulmonary artery smooth muscle cell proliferation

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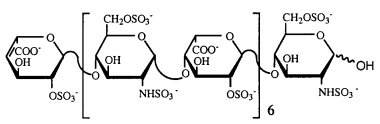
Heparin has a wide range of important biological activities including inhibition of pulmonary artery smooth muscle cell proliferation. In an attempt to understand the minimum size of heparin glycosaminoglycan chain essential for heparin-like activity, porcine intestinal mucosal (PIM) heparin was partially depolymerized with heparinase and fractionated to give different size oligosaccharides. The structure of these oligosaccharides was fully characterized by two dimensional proton NMR. These oligosaccharides (4-mer to 18-mer) were assayed for antiproliferative effects on cultured bovine pulmonary artery smooth muscle cells (BPASMC). The 14-, 16-, and 18-mers showed the same growth inhibition effect on BPASMC as PIM heparin. These data suggest that the 14-mer is the minimum size of oligosaccharide that is essential for antiproliferative activity. The sequence of the 14-mer as determined by NMR is:



C31.2

An unusual heparan sulfate motif associated with prion lesions

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Scrapie is among a group of unusual transmissible neurodegenerative diseases characterised by the deposition of an abnormally protease-resistant isoform of the prion protein. The amyloid plaques occuring in the advanced stage of the disease are known to be rich in heparan sulfate proteoglycans. An immunohistochemical study of experimental scrapie has shown that a developmentally regulated heparan sulfate antigen, 10E4, is uniquely co-distributed with the abnormal prion protein, and appears in the earliest lesions [1]. We are investigating the carbohydrate sequence recognised by this antibody using the neoglycolipid technology in conjunction with electrospray mass spectrometry. An undersulfated heparan sulfate preparation, with strong 10E4-antigenicity, has been depolymerised with heparin lyase III, the fragments converted to neoglycolipids and examined for binding by monoclonal antibody 10E4. An antigen-positive tetrasaccharide thus identified contains a unique unsulfated motif [2] with a N-unsubstituted glucosamine in the sequence:
UA-GlcN-UA-GlcNAc.

References

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C31.3

Regulation of cell cycle and expression of proteoglycans of endothelial cells transfected with EJ-*ras* oncogene

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Fetal calf serum (FCS) and PMA (phorbol 12-myristate-13-acetate) specifically stimulate the synthesis of heparan sulfate proteoglycan (HSPG) in endothelial cells, an effect that is likely to be mediated by PKC (protein kinase C) activation. Endothelial cells derived from rabbit aorta (CLPs) were transfected with the EJ-*ras* oncogene. The transfected clone showed a deregulation of the cell cycle, becoming independent of growth factors and showing an increase in G1 phase length. We also observed a decrease in the expression of the G1 regulatory proteins, cyclins D and E. In addition, there was an increase in the expression of the HSPG present in the cell and secreted to the culture medium. The wild type endothelial cells show a decrease in the release of HSPG in the G_1-S phase transition which was not observed for the EJ-*ras* transfected cells. The results suggest that the EJ-*ras* oncogene, through the signal transduction cascade, alters the regulation of the cell cycle and the expression of proteoglycans.
Supported by UNIFESP/EPM-FADA, FAPESP, CNPq and CAPES.

Reference

Porcionatto MA *et al* (1998) *J Cell Biochem* **70:**563-572

C31.4

Evaluation of heparin-protein interactions using structurally defined oligosaccharides and surface plasmon resonance

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Heparin, a heterogeneous sulfated polysaccharide, binds to various cells and proteins, and regulates their functions. Through our work on heparin-platelet and heparin-von Willebrand Factor interactions [1, 2], it was found that the clustering of some partial oligosaccharide structure in heparin is crucial to the interaction. For clearer understanding of the interaction at the molecular level, structurally defined oligosaccharides must be used. However, for the systematic study it is very difficult to obtain sufficient amounts of such fully characterized compounds from natural sources or even by chemical synthesis. To solve this problem, we developed a novel procedure using surface plasmon resonance (SPR), where the oligosaccharide is immobilized on an SPR gold chip and can be re-used. Two strategies for the immobilization were evaluated. One is based on hydrophobic interaction and the other is based on Au-S permanent binding. In both, structurally defined oligosaccharides were efficiently attached to specifically designed linker compounds using a reductive amination reaction under optimal conditions. Using known heparin-binding proteins/peptides, the Au-S method was found to have fewer non-specific interactions and higher reproducibility.

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C31.5

Affinity photo crosslinking for the identification and isolation of heparin-binding proteins

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In the study of heparin-protein interactions, it is most important to identify which protein has the specific heparin binding affinity. Because many proteins can bind to heparin specifically or non-specifically, affinity chromatographic approaches using heparin-immobilized columns may not contribute much information. If the target is a cell-surface membrane protein, the identification is even more difficult. Accordingly, we developed a method of affinity crosslinking using a newly designed hetero-bifunctional photo crosslinking reagent, named AA-D [1]. Using this reagent, we have identified that the integrin α_{IIb}/β_3 may be functionally important heparin-binding proteins on the platelet surface [2]. AA-D was also applied to an affinity resin for the isolation of high affinity heparin-binding proteins from protein mixtures [3]: one arm of the crosslinker is allowed to react with heparin immobilized on the resin. After the affinity crosslinking step, the protein-heparin complex can be recovered by specific digestion of the immobilized heparin. This may be superior to conventional affinity chromatography, since non-crosslinked and non-specifically bound proteins can be washed out.

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C31.6

Effect of the AGE-product CML-BSA on proteoglycan biosynthesis in kidney epithelial cells

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Changes in kidney function in diabetes may be due to changes in the kidney basement membranes. Proteoglycans are important constituents of this kidney extracellular matrix. Madin Darby Canine Kidney (MDCK II) epithelial cells were cultured with either low glucose (5 mM), low glucose with 10 µg/ml of N^{ε}-(carboxymethyl)-lysine bovine serum albumin (CML-BSA) or high glucose (25 mM). After two weeks cells were labelled with either [^{35}S]sulphate or [^{3}H]glucosamine for 24 h. Labelled macromolecules were purified by preparative gel filtration and ion exchange chromatography, and isolated proteoglycans were further analyzed with analytical ion exchange chromatography, gel filtration and SDS-PAGE. We show that CML- BSA, in concentrations found in the plasma of persons with diabetes type I, reduce the proteoglycan synthesis in MDCK cells, as does treatment with 25 mM glucose. The anionic properties and level of heparan sulphate are also affected by CML-BSA treatment. Advanced glycation end products may, accordingly, promote pathological changes in the extracellular matrix of the kidneys by modulating the proteoglycan biosynthesis.

Reference

Borrebæk *et al* (2001) *Diabetologia* in press

C31.7

Location of N-unsubstituted glucosamine residues in human heparan sulfate

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Structurally variable domains of heparan sulfate (HS) chains that provide binding sites for proteins are predominantly composed of hexuronic acid and either *N*-sulfated or *N*-acetylated glucosamine (GlcN) units, along with *O*-sulfate substituents in different positions. However, recent analyses of native HS indicate that a small proportion of GlcN residues may be N-unsubstituted ($GlcNH_2$), and that such units relate to important functional properties. To locate more precisely the $GlcNH_2$ units, HS from various human tissues was depolymerized by deaminative cleavage with HNO_2, under conditions leading to selective cleavage of either *N*-sulfated or N-unsubstituted GlcN residues, and the products were reduced with NaB^3H_4. Further characterization of the resultant labeled oligosaccharides revealed that most of the $GlcNH_2$ units occupy internal positions in *N*-acetylated domains, close to adjacent *N*-sulfated structures.

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C31.8

Subcellular localization of glypican-1 in endothelial cells (ECV)

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We have previously studied the biosynthesis and turn-over of glypican-1 (Gpc-1) in ECV cells. There is extensive internalization, degradation of the heparan sulphate (HS) side-chains, recycling of the core protein and resynthesis of HS. Degradation is caused by endoheparanase and by NO-derived nitrite that cleaves at occasional $GlcNH_3^+$. Here we have used a polyclonal antiserum to Gpc-1 and a monoclonal antibody to HS-epitopes containing $GlcNH_3^+$ for immunolocalization studies. Gpc-1 protein is found in granular structures throughout the cytoplasm and at the cell surface but not in the nuclei. $GlcNH_3^+$ epitopes co-localize with Gpc-1, but are also found in the nucleoli. $GlcNH_3^+$ epitopes but not Gpc-1 are destroyed by nitrite. Gpc-1 is a potential vehicle for polyamine uptake when endogenous polyamine synthesis is inhibited. Polyamine uptake is inhibited in nitrite-deprived cells. Increased polyamine uptake results in disappearance of $GlcNH_3^+$ epitopes which can be restored by nitrite-deprivation. Free HS chains are taken up by ECV cells and transported to the nucleus. A functional interplay between Gpc-1 recycling, NO and polyamine uptake is proposed.

Reference

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C31.9

Common 2-*O*-phosphorylation of xylose and differential 4-*O*-sulfation of galactose in the glycosaminoglycan-protein linkage region of syndecan-1

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We have carried out a series of structural studies of the glycosaminoglycan (GAG)-protein linkage region, and identified novel modified structures. Interestingly, sulfated Gal residues have been demonstrated so far in the linkage region of chondroitin sulfate (CS) and dermatan sulfate (DS), but not in heparan sulfate (HS) or heparin (Hep), whereas a 2-*O*-phosphorylated Xyl residue has been found in both HS/Hep and CS/DS, indicating that the sulfate groups on the Gal residues may be biosynthetic sorting signals for CS/DS. However, it remains to be examined whether such differential and common modifications reflect indeed indispensable features of the constitutive biosynthetic machinery, or cell-, tissue- or species-dependent modifications. To clarify these issues, we analyzed in the present study syndecan-1, present on the surfaces of normal murine mammary gland NMuMG epithelial cells, which is a transmembrane hybrid proteoglycan bearing GAG side chains of HS and CS. Differential 4-*O*-sulfation of one of the galactose residues and common 2-*O*-phosphorylation of the xylose residue were demonstrated.

C31.10

The covalent complex formation of hyaluronan (HA) with heavy chains of inter-α-trypsin inhibitor family is important for its functions: Defect in SHAP-HA complex causes severe female infertility

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Hyaluronan (HA) associates with proteins and proteoglycans to form the extracellular HA-rich matrices that affect significantly cellular behaviors. So far, only SHAPs (serum derived hyaluronan-associated proteins), the heavy chains of plasma inter-alpha-trypsin inhibitor (ITI) family, have been shown to bind covalently to HA. The physiological significance of such a unique covalent complex has been unknown but is of great interest because ITI is abundant in plasma, and the SHAP-HA complex is formed wherever HA meets plasma. We abolished the formation of the SHAP-HA complex in mice by targeting the gene of bikunin, the light chain of the ITI family, which is essential for the biosynthesis of ITI family and, consequently, for the SHAP-HA complex formation. The bikunin-null showed a severe female infertility. Histological examination revealed a defect in the formation of the cumulus HA-rich matrix during the preovulatory cumulus oophorus expansion. The cumulus masses of ovulated oocytes are loose, and were easily disassociated. The denuded oocytes remained unfertilized. Biochemical studies revealed that the formation of the unstable cumulus matrix was due to the impaired formation of the SHAP-HA complex in the absence of bikunin. Intraperitoneal administration of ITI fully rescued the defects. We conclude that the SHAP-HA complex but not HA is essential for the functional cumulus oophorus matrix in fertilization *in vivo*.

C31.11

The comparison of ability to exert fibrinolytic and anti-coagulant activities of chondroitin sulfate E (CSE) and fucose-branched glycosaminoglycan possessing the same core structure with CSE

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Though glycosaminoglycans (GAGs) such as heparin and dermatan sulfate have been reported to enhance the activities of the proteins related to blood coagulation and fibrinolysis, almost no reports have appeared on CSE and GAG derived thereof. Therefore, we compared the ability of these GAGs to enhance tissue plasminogen activator (t-PA) and urinary plasminogen activator (u-PA) mediated plasminogen activities as well as that to enhance heparin cofactor II (HCII) activity *in vitro*. CSE markedly enhanced t-PA and u-PA mediated plasminogen activities. This ability of CSE gradually decreased when it was digested with chondroitinase ABC, but the specific disaccharide unit ΔHexAβ1-3GalNAc(4S,6S) still showed significant ability. The fucose-branched GAG possessing the same core structure with CSE hardly showed this ability. On the contrary, CSE and the fucose-branched GAG showed the similar abilities to enhance HCII activity. These results suggested that for the ability of PA mediated plasminogen activation of these GAGs, a linear structure including GlcAβ1-3GalNAc(4S,6S) is essentially required and a branching structure abolishes the ability and that, on the contrary, for HCII activation, GlcAβ1-3GalNAc(4S,6S) is structurally important and a branching structure does not affect the ability.

C31.12

Glycotechnological preparation of chondroitin sulfate E oligosaccharides and their affinity to collagen V

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Glycosaminoglycan (GAG)-extracellular matrix protein interactions play important roles in cell adhesion and extracellular matrix assembly. We found that chondroitin sulfate E has specific affinity for type V collagen [1], but structural requirements for collagen V-chondroitin sulfate E binding have not been well characterized. Since chondroitin sulfate E has a variety of structures, it is likely that some specific feature of its structure is necessary for binding to collagen V. In the present study, the structural requirements for interaction with collagen V were characterized. Using the enzymatic reconstruction methods [2,3], we synthesized natural and artificial GAG oligosaccharides with two or three kinds of disaccharide units, such as GlcA-GlcNAc, GlcA-GalNAc, GlcA-GalNAc4S, GlcA-GalNAc6S, IdoA-GalNAc, or GlcA-GalNAc4,6-diS, and used them as model oligosaccharides. The binding of these oligosaccharides to collagen V was examined using biosensor-based surface plasmon resonance (SPR). As a result, it was found that GlcA-GalNAc4,6-diS (E unit) was essential for collagen V binding and the minimum size of oligosaccharide required for the binding was octasaccharide having three continuous E units at the nonreducing site. Therefore, it is suggested that a specific domain containing E unit structure is involved in extracellular matrix formation.

References

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[2] Takagaki K *et al* (2000) *Biochem Biophys Res Commum* **270**:588-593
[3] Takagaki K *et al* (2000) *J Biochem* **127**:695-702

C31.13

Human hyaluronidase polymorphism, evidence for conserved hyaluronidase potential N-glycosylation sites in mammalian and non-mammalian species

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A number of properties of human hyaluronidases in somatic tissues and in body fluids were studied. When analyzed on a PAGE-hyaluronan gel, liver and placenta exhibited 7 hyaluronidase forms while plasma and synovial fluid presented 3 and 2 forms, respectively. Sialidase treatment decreases the number of forms to 3 in liver, 2 in placenta and to a slow basic form in serum. Plasma and placental hyaluronidases remain fully active after thermal treatment but desialylated hyaluronidase is inactivated slowly in plasma, and fast in placenta suggesting a higher overall glycosylation of the plasma enzyme. Potential N-glycosylation sites were searched in the amino acid sequences in hyaluronidases in human and other mammalian and non-mammalian species. A potential N-glycosylation site was observed at the same position in human plasma, human lysosomes, human Hyal-4 and Hyal-p1. The same site was also present in mouse plasma, mouse lysosomes, rat lysosomes, frog liver, wasp, hornet and honeybee venom, and *C. elegans*. However, this site was absent in human Hyal-3, in human meningioma and in all sperm hyaluronidases examined. A second potential N-glycosylation site is also present in all hyaluronidases with an identical pattern (Asn-Val-Thr) except for those of lysosomal origin (Asn-Val-Ser). Site 2 is absent in honeybee venom and in *C. elegans*. Such conserved sites strongly suggest that they may represent N-glycosylation sites.

Reference

Fiszer-Szafarz B *et al* (2000) *J Biochem Biophys Meth* **45**:103-116

C31.14

Characterization of glucuronyl C5-epimerase in biosynthesis of heparin and heparan sulfate glycosaminoglycans

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The murine gene for the glucuronyl C5-epimerase involved in heparan sulfate biosynthesis was cloned, using a previously isolated bovine lung cDNA fragment as probe. The ~11 kb mouse gene contains 3 exons from the first ATG to stop codon and is localized to chromosome 9. Southern analysis of the genomic DNA and chromosome mapping suggested the occurrence of a single epimerase gene. Based on the genomic sequence, a mouse liver cDNA was isolated that encodes a 618 amino acid residue protein, thus extending by 174 N-terminal residues the sequence deduced from the (incomplete) bovine cDNA. Comparison of murine, bovine and human epimerase cDNA structures indicated 96-99% identity at the amino acid level. A cDNA identical to the mouse liver species was demonstrated in mouse mast cells committed to heparin biosynthesis. These findings suggest that the iduronic acid residues in heparin and heparan sulfate, despite different structural contexts, are generated by the same C5-epimerase enzyme. The catalytic activity of the recombinant full-length mouse liver epimerase, expressed in insect cells, was found to be >2 orders of magnitude higher than that of the previously cloned, smaller bovine recombinant protein. The ~52 kDa, similarly highly active, enzyme originally purified from bovine liver was found to be associated with a ~22 kDa peptide generated by a single proteolytic cleavage of the full-sized protein.

References

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Li J-P *et al* (1997) *J Biol Chem* **272:**28158-28163

C31.15

Disaccharides of keratan sulfate induces cell death of lymph nodes T cells by reducing IL-12 production in MRL-*lpr/lpr* mice

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It has been shown that disaccharides of heparin or heparan sulfate inhibits TNF-α production. This suggests that oligosaccharides of glycosaminoglycans play roles in the regulation of cytokine production. In the present study, keratan sulfate disaccharides, Gal(6-SO_4)-GlcNAc(6-SO_4): [L4] was injected into MRL-*lpr/lpr* (MRL-*l*) mice which develope an autoimmune disease accompanied by the lymphoaccumulation. The treatment of L4 suppressed the increase in lymph nodes weights as well as plasma IL-12 level. Increase in apoptotic cells was observed in the lymph nodes of the MRL-*l* mice treated with L4. Moreover, the treatment of L4 suppressed IL-12 production in cultured mice peritoneal macrophages stimulated with lipopolysaccharides and IFN-β. It is well known that lympho-accumulation occurs in MRL-*l* mice because T cells cannot be deleted due to the *Fas* mutation. We have previously shown that IL-12 suppresses cell death of T cells to enhance the lymphoaccumulation in MRL-*l* mice. These events suggest that L4 induces the cell death of T cells followed by the reduced lymphoaccumulation by suppressing the production of IL-12 in MRL-*l* mice.

Reference

Xu H *et al* (2001) J Autoimmun **16:**87-95

C32. Sialic acids

C32.1

Characterization of the sialate-9-*O*-acetyltransferase (9-OAT) activity from human colonic mucosa

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The sialic acids in human colonic mucins are highly *O*-acetylated, however, little is known about the underlying enzymatic activity required for *O*-acetylation in this tissue. Here, we report on the characterization of the microsomal 9-OAT using CMP-Neu5Ac, the most efficient acceptor substrate of all those tested (including Neu5Ac, edible bird nest substance, de-*O*-acetylated human colonic mucins and endogenous glycoconjugates). The enzyme activity was found to be optimal at 37 °C, with a pH optimum of 7.0. Activity was also found to be dependent on protein, CMP-Neu5Ac and acetyl-coenzyme A concentration, as well as membrane integrity. The enzyme activity could be inhibited by coenzyme A. In addition, enzyme activity was found to be localized in the Golgi-enriched membrane fraction. Using CMP-Neu5Ac, the neo-*O*-acetylated products formed via the action of 9-OAT were verified with the aid of the chromatographic and enzymatic techniques. The main product was 9-*O*-acetylated Neu5Ac, while a significant amount of oligo-*O*-acetylated Neu5Ac was also detected. Furthermore, the utilization of CMP-Neu5Ac as the acceptor substrate was confirmed by the isolation and characterisation of neo-CMP-Neu5,9Ac_2 following ion-exchange chromatography. The conclusive demonstration for the formation of CMP-Neu5,9Ac_2 was afforded by the ability of neo-radiolabeled CMP-Neu5,9Ac_2 to act as a donor substrate for the sialyltransferases from human colonic Golgi apparatus and α-2,6-sialyltransferase from rat liver.

C32.2

Characterization of the membrane-bound sialate 7(9)-*O*-acetyl-transferase from bovine submandibular glands

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The occurrence and identification of *O*-acetylated sialic acids has been demonstrated in many animal tissues and some microorganisms [1]. These ester residues most frequently occur on the glycerol side chain of sialic acids and can form mono- to tri-*O*-acetylated species [1].
The transfer of acetyl groups into endogenous acceptors present in the microsomes isolated from bovine submandibular glands was found to be optimal at 37 °C, and had a pH optimum of 7.5 with an apparent K_M for AcCoA of 8μM. The incorporation of [^{3}H]acetate was also dependent on time, protein concentration and was inhibited by CoA in a concentration-dependent manner.
The characterization and quantification of purified sialic acids from microsomes was achieved by thin-layer chromatography and high-performance liquid chromatography. Work is currently ongoing to further elucidate the mechanism by which the enzymatic *O*-acetylation reaction takes place in bovine submandibular glands. Many questions in this system still remain unsolved. For instance, which position of sialic acid at C-7 or C-9 is the primary attachment site? Is there more than one enzyme involved? Does there exist an isomerase and how is the *O*-acetyltransferase linked to a putatitve acetyl-CoA transporter?

Reference

[1] Schauer R, Kamerling JP (1997) In: Glycoproteins (Montreuil J, Vliegenthart JFG, Schachter H, eds) pp 243-402, Elsevier, Amsterdam

C32.3

Molecular cloning and developmental expression of two α2,8-polysialyltransferases in rainbow trout ovary

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Rainbow trout egg polysialoglycoprotein (PSGP) is the first example of polysialylated glycoprotein in vertebrates. The α2,8-linked oligo/polysialic acid (oligo/polySia) chains are attached to the O-glycans of PSGP. Based on developmental changes of the glycan structures of PSGP during oogenesis, it is proposed that three sialyltransferase activities are involved in the biosynthesis of oligo/polySia chain of PSGP: (i) α2,6-sialyltransferase, which transfers α2,6-linked Sia residue to the proximal GalNAc residue; (ii) α2,8-sialyltransferase, which transfers α2,8-linked Sia residue to the α2,6-linked Sia residue; (iii) α2,8-polysialyltransferase, which catalyzes the elongation of α2,8-linked oligo/polySia chain on the Sia residue transferred by the α2,8-sialyltransferase [1]. In this study, the cDNAs encoding the polysialyltransferase PST and STX homologues have been cloned from rainbow trout ovary and other tissues. In Northern blot analysis, the PST homologue is expressed in several tissues, especially in kidney, spleen and ovary, while the STX homologue is specifically expressed in embryos and ovary. The expression level of mRNA of STX homologue is elevated at later stages during oogenesis, concomitant with extensive polysialylation of PSGP.

Reference

Kitazume *et al* (1994) *J Biol Chem* **269**:10330-10340

C32.4

Domain structure of UDP-GlcNAc 2-epimerase/ManNAc kinase

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UDP-GlcNAc 2-epimerase/ManNAc kinase catalyzes the first two steps of sialic acid biosynthesis in mammals. Sequence similarities to other enzymes suggested that the bifunctional enzyme consists of separate catalytic domains. For functional characterization in a first approach site-directed mutagenesis was performed on different conserved amino acids [1]. Mutants as well as the wild-type enzyme were expressed in insect Sf9 cells using the baculovirus system. Point mutations of five histidines in the N-terminal half led to a loss of epimerase activity with almost unchanged kinase activity. On the other hand mutations in the C-terminal half of the protein caused a loss of kinase activity while epimerase activity still remained. These results suggest two active sites working quite independently.
Several deletion mutants were constructed for a separate expression of the two postulated domains. Introduction of stop-codons in the C-terminal part of the enzyme resulted in expression of the epimerase domain with only parts of the kinase domain. Specific cleavages in the 5`-part of the cDNA led to expression of enzymes lacking parts of the epimerase domain. Results about enzyme activities and structures of the deletion mutants will be presented.

Reference

[1] Effertz K *et al* (1999) *J Biol Chem* **274**:28771-28778

C32.5

β-Galactoside α-2,6-sialyltransferase (ST6Gal I) expression effects adhesion and invasion of breast carcinoma cells

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Tumor-associated alterations of cell surface glycosylation play a crucial role for the adhesion and metastasis of carcinoma cells. The aim of the present study was to examine the adhesion and invasion properties of tumor cells with different α-2,6-sialylation. To this end, mammary carcinoma cells MDA-MB-435 were sense-transfected with sialyltransferase ST6Gal I cDNA or antisense-transfected with different parts of the ST6Gal I sequence inserted into pcDNA 3.1 vector. In another approach, incubation of soluble antisense-oligodeoxynucleotides (antisense-ODN) was applied to inhibit ST6Gal I enzyme activity. Sense-transfectants showed an enhanced ST6Gal I mRNA expression and enzyme activity, increased binding of the lectin SNA, specific for α-2,6-sialylated oligosaccharides and more cell surface-bound sialic acid. Antisense-transfections resulted in a lower mRNA expression, less enzyme activity, less SNA-reactivity and reduction of cell surface sialic acid. Sense-transfected clones adhered particularly strong to collagen IV, showed reduced cell-cell adhesion and an enhanced invasion capacity, whereas antisense-transfected clones adhered less to collagen IV but showed strong homotypic cell-cell adhesion. Treatment of DAUDI B-lymphoma cells with soluble antisense-ODN significantly reduced enzyme activity and SNA-binding. Cell surface α-2,6-sialylation is of high relevance for the adhesive and invasive properties of tumor cells. Inhibition of ST6Gal I by antisense-ODN might be a way to reduce the metastatic capacity of carcinoma cells.

C32.6

Molecular cloning and substrate specificity of the vertebrate *N*-acetylneuraminic acid 9-phosphate synthases

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A cDNA clone for the mouse homologue [1] and three cDNA clones of the rainbow trout homologue of *Escherichia coli N*-acetylneuraminic acid (Neu5Ac) synthase (*neuB* gene product) were cloned by the PCR-based method. The mouse and trout homologues consist of 359 and 362 amino acids. The trout cDNA sequences display >99% identity among those three clones and 77% identity to that of the mouse homologue. Three highly conserved amino acid sequence motifs are found in these mouse and trout homologues and the known bacterial Neu5Ac synthases. The recombinant mouse and trout homologues which were expressed in *E. coli* exhibited the Neu5Ac 9-phosphate (Neu5Ac-9-P) synthase activity, which catalyzes condensation of phosphoenol-pyruvate (PEP) and *N*-acetylmannosamine 6-phosphate to synthesize Neu5Ac-9-P, but not the Neu5Ac synthase activity. Thus, these vertebrate enzymes are identified as the Neu5Ac-9-P synthase. These Neu5Ac-9-P synthases require divalent cations (Mg^{2+}, Mn^{2+}). Notably, they can not catalyze the synthesis of deaminoneuraminic acid 9-phosphate (KDN-9-P) from PEP and Man 6-phosphate effectively, suggesting that these enzymes appear not to be involved in the synthesis of KDN.

Reference

[1] Nakata D *et al* (2000) *Biochem Biophys Res Commun* **273**:642-648

C32.7

Uptake and metabolic incorporation of unnatural sialic acids by eukaryotic cells

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Sialic acids bound to glycoconjugates play a crucial role in cell-cell and cell-pathogen interactions and are therefore of interest for detailed structure-function studies. We recently demonstrated the efficient uptake and incorporation of free *N*-acetylneuraminic acid by hyposialylated human cell lines [1]. Here we provide evidence that these cells can also incorporate a variety of unnatural sialic acids. For a C5 and a C9 substituted analogue the identity between added and incorporated sialic acids was confirmed by HPLC. The incorporation of specific sialic acid analogues had striking functional consequences for the sialic acid-dependent infection of BJA-B cells by the B-lymphotropic papovavirus. This novel system allows for a versatile and efficient biosynthetic modulation of surface sialylation in living cells. It complements the established usage of unnatural sialic acid precursors and increases the number of potential modification sites.

Reference

[1] Oetke C *et al* (submitted)

C32.8

5-Azido sialic acid as new glycosylation reagent - An excellent α-selectivity donor

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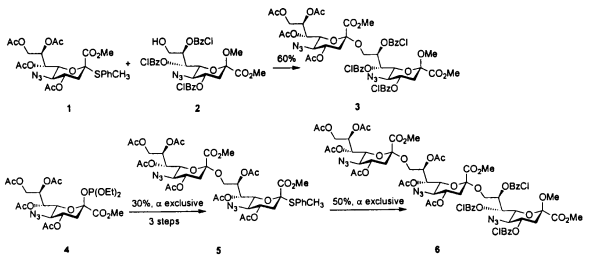
Sialylation with α-selectivity is one of the most challenging problems in the synthesis of oligosaccharides. We report here two novel sialyl donors: thiol donor **1** and phosphite **4** by modification of the NHAc group to an azido group. The sialylation of **1** with **2** gave with exclusive α-selectivity α-2,9 sialic acid dimer **3** in 60% yield. By using an orthogonal glycosylation strategy, we have successfully synthesized α-2,9 sialic acid trimer **6**.



C32.9

Up-regulation of membrane-associated ganglioside sialidase in human colon cancer and its involvement in apoptosis suppression

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Membrane-associated ganglioside sialidase has been suggested to participate in regulation of cell proliferation and differentiation by modulating gangliosides on plasma membranes. To obtain the functional evidence in molecular basis, we previously cloned cDNAs of human and mouse ganglioside sialidases. The purpose of this study was to determine whether the expression of ganglioside sialidase is altered in human colon cancer and how it influences malignant property. Quantitative RT-PCR, in situ hybridization, immuno-histochemistry and sialidase activity assays were used to assess the expression of ganglioside sialidase in colon cancer tissues. Levels of the mRNA were increased by >20-fold in colon cancer tissues compared to adjacent nontumorous mucosa. In situ hybridization and immunohistochemical analysis exhibited that the sialidase was expressed in adenocarcinoma cells of the colon. In cultured human colon cancer cells, the levels of the sialidase were down-regulated in the process of differentiation and of apoptosis induced by sodium butyrate. Transfection of this gene into the cancer cells inhibited butyrate-induced apoptosis and differentiation, indicating increased expression in colon cancer cells leads to protecting tumor cells from programmed cell death. Taken together, these results suggest that the sialidase may be a target for the prevention or treatment of colon cancer.

C32.10

Identification of a new diSia-containing glycoprotein (adipo Q) in bovine and mouse sera

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α2→8-Linked polySia chain with 10-55 Sia residues is well known to be a regulatory molecule of cell-cell communication during development and cell differentiation. Recently, we have demonstrated that the new class of sialyl epitopes consisting of di- and oligosialyl structures with up to 7 Sia residues occurs in glycoproteins more frequently than heretofore-recognized [1]. In this study, we identified adipo Q, an adipocyte specific glycoprotein involved in energy regulator, as a new member of oligoSia-glycoprotein in fetal bovine serum by chemical and immunochemical methods. We also identified that mouse adipo Q is a diSia-containing glycoprotein in serum as well as in adipocyte-differentiated 3T3-L1 cells. To gain an insight into the biosynthetic pathway of α2,8-linked diSia structure on adipo Q, we analyzed the expression level of known α2,8-sialyltransferases responsible for the formation of α2,8-linked Sia residues in glycoprotein(s) and glycolipid(s) by RT-PCR. Of these enzymes, ST8Sia III was only found to be expressed in 3T3-L1 cells during the adipocyte differentiation, suggesting that ST8Sia III is involved in the synthesis of diSia glycotope on adipo Q.

Reference

[1] Sato C *et al* (2000) *J Biol Chem* **275**:15422-154311

C32.11

Mechanism of cooperative polysialylation of NCAM by two polysialyltransferases, PST (ST8Sia IV) and STX (ST8Sia II)

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ST8Sia II and IV are polysialyltransferases that form polysialic acid (PSA), a homopolymer of α2,8-linked SA, on neural cell adhesion molecule (NCAM). We previously reported that these two enzymes can synthesize PSA on NCAM in a synergistic manner. However, it is still unknown how these two enzymes work together and how the amount or length of polysialylation is regulated during PSA synthesis. Here we approach these problems by *in vitro* polysialyltransferase assay using radiolabeled CMP-SA and NCAM•Fc mutated at N-glycosylation sites or synthetic oligosaccharides as acceptors. First, we demonstrated that cooperative PSA synthesis depends on each enzyme's different preference for different N-glycosylation sites. Next, we found that pre-existing PSA does not inhibit *de novo* polysialylation; secondary polysialylation reaction takes place in the same core glycan which had PSA on one branch. Multiple SA can be added more efficiently on mono- and oligo-SA but PSA was not utilized as acceptor, suggesting long SA is unlikely elongated by polysialyltransferases. HPLC analysis using Mono-Q anion exchange column demonstrated that free branch(es) on the core N-glycan of PSA-NCAM was used as a second polysialylation site. These results, combined together, suggest that polysialyltransferases are capable to produce large amount of PSA by using different N-glycosylation sites on NCAM and by using different antennas of the same N-glycan. (Supported by CA33895).

C32.12

Mutational study on the impact of N-glycans for enzymatic activities of mammalian polysialyltransferases

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Poly-α2,8-*N*-acetylneuraminic acid (polysialic acid, PSA) is a specific and highly regulated posttranslational modification of the neural cell adhesion molecule (NCAM). PSA modulates the NCAM binding properties and plays a crucial role in neural development, neural regeneration and plastic processes in the vertebrate brain [1]. The polysialyltransferases ST8SiaII and ST8SiaIV are independently able to synthesize PSA on NCAM. In addition, they catalyze autopolysialylation, an unusual self-modification that occurs on N-glycans of the enzymes. Using site directed mutagenesis, N-glycosylation sites were eliminated in various combinations to analyze the role of individual N-glycans for enzymatic activities. In ST8SiaIV a single N-glycosylation site could be identified (Asn-74), which is sufficient for enzymatic activity, while for ST8SiaII the presence of two N-glycans (Asn-89 and -219) is of importance. Loss of autopolysialylation activity due to elimination of specific N-glycosylation sites did always lead to a complete loss of enzymatic activity, including NCAM-polysialylation, providing evidence for a tight functional link between auto- and NCAM-polysialylation.

Reference

[1] Mühlenhoff *et al* (1998) *Curr Opin Struct Biol* **8**:558-564

C32.13

Identification of the nuclear localisation signal in the murine CMP-*N*-acetylneuraminic acid synthetase

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Sialic acids occur as terminal sugars with great variability on many glycoproteins and glycolipids of the cell surface. Due to this exposed position sialic acids contribute to the regulation of cell-cell interactions (for review see [1]). To serve as substrates for sialyltransferases sialic acids need to be activated to CMP-sialic acids. This reaction is catalysed by the CMP-*N*-acetylneuraminic acid synthetase. While all other sugar activating enzymes have been found in the cytoplasm, the CMP-Neu5Ac syn is located in the cell nucleus (for review see [2]). This unusual localisation of the enzyme has been investigated for more than 30 years but still provides an unresolved problem. We recently isolated the cDNA of the murine CMP-Neu5Ac syn [3]. To evaluate the functional competence of the three putative nuclear localisation signals the intracellular localisation of eGFP fusion proteins was analysed by indirect immunofluorescence microscopy. By site-directed mutagenesis cytoplasmic variants of the CMP-Neu5Ac syn have been generated and enzymatic activity has been analysed. Our results show that the enzymatic activity of the CMP-Neu5Ac syn does not depend on nuclear localisation.

References

[1] Kelm S *et al* (1997) *Int Rev Cytol* **175:**137-240
[2] Kean E *et al* (1991) *Glycobiology* **5:**441-447
[3] Münster AK *et al* (1998) *Proc Natl Acad Sci USA* **95:**9140-9145

C32.14

Targeted mutagenesis of cytidine 5′-monophosphate *N*-acetylneuraminic acid synthetase in the mouse

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The cytidine 5′-monophosphate *N*-acetylneuraminic acid synthetase (CMP-Neu5Ac Syn) catalyses the activation of *N*-acetylneuraminic acid to CMP-Neu5Ac. CMP-Neu5Ac provides a substrate for Golgi sialyltransferases and is a prerequisite for the synthesis of sialylated glycoconjugates. As terminal residues of cell surface glycans, sialic acids are involved in the formation of cellular recognition structures, regulate cell-cell interactions, play a role in neuronal plasticity and in tumor growth and metastasis (for review see [1,2]). We have recently cloned the murine CMP-Neu5Ac Syn gene [3]. In contrast to other sugar activating enzymes, the CMP-Neu5Ac Syn is located in the nucleus. This nuclear localisation and the enzymatic activity are dependent on the presence of a particular nuclear localisation signal. We have started conditional targeted mutageneses using the cre/loxP system to abolish enzymatic activity and to direct a cytoplasmic localisation of an active enzyme respectively. These approaches will enable us to investigate ontogenesis in mice, in which the formation of sialoconjugates can be interrupted. Furthermore it will provide the tool to investigate the importance of a nuclear localisation.

References

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[3] Muenster AK *et al* (1998) *Proc Natl Acad Sci USA* **95**:9144-45

C32.15

Cellular localisation of CMP-Neu5Ac hydroxylase

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N-Glycolylneuraminic acid (Neu5Gc) is an abundant sialic acid in many deuterostome animals. The biosynthesis of Neu5Gc is carried out by cytidine-5'-monophosphate-*N*-acetylneuraminic acid (CMP-Neu5Ac) hydroxylase (EC 1.14.13.45) giving rise to CMP-Neu5Gc. This enzyme is a monooxygenase which requires the membrane-bound cytochrome b_5-system for delivery of electrons from NADH. In contrast to all other known cytochrome b_5-dependent enzymes, mammalian CMP-Neu5Ac hydroxylase is a cytosolic protein, as shown by tissue fractionation and cDNA sequencing. However, immunohistochemical studies on porcine lymphocytes revealed an association of the hydroxylase with the cytosolic face of several organelles. In contrast to the mammalian hydroxylase, CMP-Neu5Ac hydroxylase from echinoderms is membrane-associated. Using cDNA cloning and sequencing it was found that a large segment of the enzyme from the starfish *Asterias rubens* is highly homologous to the mammalian hydroxylase. However, the echinoderm enzyme possesses an additional C-terminal sequence, including a stretch of hydrophobic amino acids which may form a membrane-binding domain. This was confirmed by transient expression of a chimeric protein, consisting of the complete soluble mouse hydroxylase and the putative membrane-binding domain of the starfish enzyme, in COS cells. The possible functional consequences of the differential localisation of the mammalian and echinoderm enzymes are discussed.

C32.16

Molecular mechanism of polysialylation: lack of evidence for auto-polysialylation during embryonic chick brain development (ECB)

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Background: To understand the mechanism of polysialylation it is important to determine if "autopolysialylation" of the polysialyltransferases STX and PST is a physiologically relevant event during normal development. To test this hypothesis, we have developed anti-STX antibodies, and have used these in combination with anti-polySia mAbs and Endo-N to study the developmental expression of STX in ECB.
Results: (1) Anti-STX detected two bands of immunoreactivity with the M_r expected for a monomer (~45-50 kDa) and a dimer (~90-100 kDa). Neither band was sensitive to Endo-N, implying that no Sia chains with DP of ~5 or greater were present on the enzyme. (2) Unexpectedly, maximal expression of the STX monomer appeared earlier in development (E5-E8) than the dimer (E8-E14).
Conclusions: (1) STX is not autopolysialylated during ECB development. Thus, the autopolysialylation observed in transiently transfected cell lines may not be physiologically relevant during neural development. Rather, it may be the consequence of transfecting high levels of these STX/PST genes into cell lines that do not normally express polySia. (2) Given the close temporal correlation between expression of the STX dimer, polyST activity and polySia expression, these results suggest that the dimer may be the more active form of the membrane-bound STX. (Supported by NIH Grant GM55703).

C32.17

Substrate- and inhibitor-studies on a recombinant trans-sialidase from *Trypanosoma cruzi*

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Trans-sialidases (TS) catalyse the transfer of α2,3-linked sialic acid directly to terminal β-galactose-containing acceptors forming a new α2,3-linkage [1]. These enzymes are believed to play an important role in maintaining the pathogenicity of trypanosomes [2]. Because of the possibility to use TS for the biotechnological sialylation of carbohydrates and the aim to find potent TS-inhibitors for the therapy of Chagas´ disease, in this work several donors, acceptors and inhibitors - chemically synthesized compounds as well as substrates from natural sources - have been tested with a recombinant trans-sialidase from *Trypanosoma cruzi*. The assay used is a modified non-radioactive test from Engstler *et al* [3], which was further developed in order to rapidly screen larger sample amounts.

References

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C32.18

Sialic acid species as a determinant of the host range of influenza A viruses

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Sialic acids (SA) in host cell receptors are widely distributed in animals, however, the molecular species and the sialyl linkages vary among animal species. Previous studies have shown that the sialyllacto/sialyl neolacto-series sugar chains, SAα2-3(6)Galβ1-3(4)GlcNAcβ1-, in glycoproteins and glycolipids are the functional receptor sugar chains for influenza A and B viruses from humans and animals. Amino acid substitutions in hemagglutinin (HA), the glycoprotein responsible for receptor binding of influenza viruses, have resulted in changing receptor specificity for the molecular species (Neu5Ac, Neu5Gc) as well as sialyl linkage (SA2-3Gal, SA2-6Gal). The host range is influenced by host cell receptors; the Neu5Gc2-3Gal moiety present on crypt epithelial cells of duck colon have been shown to play an important role in the enterotropism of avian influenza viruses. In addition, a virus with an HA recognizing the Neu5Ac2-6Gal but not Neu5Ac2-3Gal or Neu5Gc2-3Gal, failed to replicate in horses, while one with an HA recognizing the Neu5Gc2-3Gal moiety replicated in horses. The abundance of the Neu5Gc2-3Gal moiety in epithelial cells of horse trachea supports that recognition of this moiety is critical for viral replication in horses. Thus, substantial evidence suggesting the significance of the molecular species and linkage in the host range of the influenza. Here we report the biological role of receptor sialyl sugar chains in host range determination of influenza A viruses.

Reference

Suzuki Y *et al* (2000) *J Virol* **74**:11825-11831

C32.19

Identification and characterisation of an enzymatic sialate-de-*N*/re-*N*-acetylation reaction in bovine submandibular glands

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Sialic acids comprise a family of over 40 naturally occurring derivatives of the nine carbon sugar, designated neuraminic acid (Neu). The largest structural variations of sialic acids occurs at carbon 5, with *N*-acetylation of the amino group at this position being the most common. Further studies, however, have also revealed the presence de-*N*-acetylated-gangliosides [1,2] (gangliosides which possess a free amino group at C-5 of Neu). These molecules have been found to exist in certain tumour cells [1,2], and have been shown to enhance tyrosine phosphokinase activity and cellular proliferation [3].
The enzymatic de-*N*/re-*N*-acetylation of Neu at C-5 was first observed in the gangliosides of human melanoma cells [1]. Here we report on the characterisation of an enzymatic sialate de-*N*/re-*N*-acetylation reaction in the cytosolic fraction from bovine submandibular glands that catalyses the de-*N*-acetylation of exogenous free *N*-acetylneuraminic acid (Neu5Ac), followed by re-*N*-acetylation of Neu utilising [^{3}H]AcCoA as the acetyl donor substrate.

References

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C32.20

Purification and initial characterisation of trans-sialidase from *Trypanosoma congolense*

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Sialidases hydrolyse glycosidically linked sialic acid, in which a water molecule is the acceptor. In contrast, trans-sialidases (TS) catalyse the transfer of, preferably, an α2,3-carbohydrate-linked sialic acid to another carbohydrate forming a new α2,3-glycosidic linkage to galactose or *N*-acetylgalactosamine. TS was first described in the American species *Trypanosoma cruzi* [1]. Trypanosomes do not synthesise sialic acid [2], but utilise TS to acquire it from the mammalian host. Sialic acid transferred to surface glycoproteins may be required for the survival and pathogenicity of the parasites inside the vector and the host [3]. Here we report on the cultivation of *Trypanosoma congolense* and present purification, characterisation and inhibition experiments of the TS from culture medium. In addition we outline a molecular biological approach, which is hoped, will lead to the expression of recombinant trans-sialidase from African trypanosomes.

References

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C32.21

Molecular mechanism of polysialylation: Deletion mutagenesis to determine the function of the conserved basic amino acid sequence unique to the α2,8-polysialyltransferases

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Background: In contrast to the α2,3-, α2,6- and α2,8-monosialyltransferases (monoSTs), the α2,8-polySTs (STX and PST) contain a conserved, uniquely extended basic amino acid region (pI 11.6-12.0) of 31 or 32 residues immediately upstream of sialylmotif S. We have hypothesized that this positively charged region may function in the processive mechanism of chain polymerization by allowing nascent polySia chains to remain tethered to the enzyme during the repetitive addition of each new Sia residue to the non-reducing terminus of the growing chain.
Experimental Strategy: To test this hypothesis, the entire 32 mer was deleted from PST. In addition, deletion mutants lacking 10 or 15 amino acids residues from either the N- or C-terminal region of the 32 mer were constructed.
Results: CMP-[^{14}C]Neu5Ac was used to determine the level of mono- and α2,8-polyST activity in membranes from wild type and mutated PST transfected Cos-1 cells, as previously described. Our results showed nearly complete loss of polyST activity in the deletion mutants, and a lesser attenuation of monoST activity.
Conclusions: Our initial findings support the hypothesis that the 32 amino acid sequence in PST may play a critical role in polySia chain synthesis. Further studies will be required to elucidate the exact role of this structurally unique motif in polysialylation. (Supported by NIH Grant GM55703).

C32.22

Escherichia coli K92 polysialyltransferase reaction

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Escherichia coli K1 and K92 produce extracellular polysaccharides consisting of polysialic acids. *E. coli* K1 causes neonatal meningitis and urinary tract infections. *E. coli* K92 is cross-antigenic with the pathogen *Neisseria meningitidis* group C. The K92 polysaccharide has an alternating repeating unit of α(2-8) and α(2-9) sialic acid. We have recently shown that the membrane bound K92 polysialyltransferase can synthesize both linkages. This enzyme has an acceptor preference for α(2-8) sialic acid although it can elongate α(2-9). This polysialyltransferase cannot initiate polymer synthesis without externally added acceptor. In this report we present data which suggest that the initiation of polymer synthesis may require the action of other proteins encoded in the gene cluster. The ability to initiate synthesis was not restored to cells harboring the K92 polysialyltransferase by complementation with individual gene products of neuD, neuE, or kpsS. However, appropriate gene clusters of either *E. coli* K1 or K5 restore initiation. The K5 polysaccharide is not a polysialic acid. The molecular weight of the activity required for elongation of externally added polysaccharide was compared by radiation target analysis with that required for initiation of synthesis. The results suggest that both activities are the same target size implying that no additional proteins are required to catalyze the initiation reaction. Additional protein products of the K92 gene cluster maybe required for formation of endogenous acceptor.

C32.23

An ultrasensitive chemical method for polysialic acid analysis

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An ultrasensitive method to analyze polysialic acid (polySia) chains using fluorescence-assisted HPLC was developed. The new method is an utmost improvement of our earlier method in which the reducing terminal Sia residues of a homologous series of oligo/polySia produced during reaction were simultaneously labeled with a fluorogenic reagent, 1,2-diamino-4,5-methylenedioxybenzene (DMB) under acidic conditions. To establish the optimal reaction condition under which high proportions of the parent polySia retained while keeping satisfactorily high derivatization rate, we first studied the stability of authentic oligo/polySia in the acid (0.02 M TFA) used for DMB derivatization under various conditions of reaction time and temperature using high performance anion exchange chromatography with pulsed electrochemical detection (HPAEC-PED) for the analysis of reaction products. Using the new methods, baseline resolution of $(Sia)_n$ with DP (degree of polymerization) 2 to ca 90 with a detection threshold of 1.4 femtmole per resolved peak was achieved for commercial colominic acid. The new method was verified to be also applicable to DP analysis of polySia chains linked to core glycan chains. We will show the results of polySia analysis of embryonic NCAM using the new method comparing with those using HPAEC-PED, and the application of the new method to polySia analysis in crude homogenate of 10–100 mg of cells and tissues. (Supported by NSC 90-2311-B-001-021 to S.I. and NHRI-EX90-8805BP toY.I.).

C32.24

Developmental profile of NCAM glycoforms with varying degree of polymerization of polysialic acid chains

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Expression of polysialic acid (polySia) is a unique mechanism of regulating the adhesive property of the neural cell adhesion molecule (NCAM) in vertebrates. In higher vertebrates, polysialylation of NCAM is a process coordinated with the development of the nervous system in embryonic animals, and regeneration of neuronal tissues in adults. Recently, the regulated expression of polysialyltransferases that participate in NCAM polysialylation and the properties of the enzymes have been extensively studied. However, NCAM glycoforms with different degree of sialic acid contents have been characterized only qualitatively. More precise information on the chemical structure of glycan chains including the degree of polymerization (DP) of polySia chains, and its developmental stage-dependent variation is considered important in understanding the molecular mechanism of regulated polysialylation and fine-tuning of NCAM-mediated cell adhesion by polySia chains. Using the newly developed ultrasensitive method [Inoue S *et al* (2001), this Symposium], we revealed the presence of numerous NCAM glycoforms differing in DP of oligo/polySia chains and showed that their distribution exhibits a delicate change during development of chicken brain. We will also present the evidence showing that 2 of 3 arms of the tri-antennary N-glycan are oligo/polysialylated. (Supported by NSC 90-2311-B-001-021 to S.I. and NHRI-EX90-8805BP to Y.I.).

C32.25

A preliminary study of a putative sialic acid-recognising enzyme from *Mycobacterium smegmatis*

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Tuberculosis (TB) is a chronic bacterial disease causing disability and death worldwide. The causative agent, *Mycobacterium tuberculosis* is a member of a genus characterized by the presence of thick waxy cell walls containing many unusual glycolipids. Intrinsic resistance to antibiotics and resistance to available agents characterize species of Mycobacteria. We have been interested in characterizing some of the enzyme activities of these bacteria for activities that may be amenable to inhibition. Mycobacterial species, *M. smegmatis* mc^2155 and *M. bovis* BCG were screened for enzymes of interest by taking cell lysates and exposing them to various substrates. Lysates were found to hydrolyse 4-methylumbelliferyl α-*N*-acetylneuraminic acid (MUN) [1]. A partially-purified cell lysate was observed to cause hydrolysis of both MUN and sialyllactose using ^{1}H NMR spectroscopy [2].

References

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C32.26

Expression and characterization of the active form of human ganglioside GM3 synthase (sialyltransferase-1/ST3GalV) in *Escherichia coli*

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Human sialyltransferase-I (hSAT-I/GM3 synthase/ST3GalV/α2,3-sialyltransferase) is the only sialyltransferase so far cloned that can catalyze the biosynthesis of ganglioside GM3 *in vivo*. In this experiment, hSAT-I lacking the cytoplasmic and transmembrane domains, could be successfully produced in a soluble form in *Escherichia coli* harboring the expression plasmid containing the *hSAT-I* cDNA. The GM3 synthetic activity was detected in both the soluble and the insoluble form of hSAT-I, which is expressed as MBP (maltose binding protein)-fusion protein. The kinetics values for the soluble form hSAT-I were similar to those for the enzymes extracted from mammalian tissues. The acceptor substrate specificity was also similar to that of the hSAT-I expressed in mammalian cells. Since, in *E.coli*, glycosylation of the expressed protein is not to be carried out, it suggested that such modification of the sialyltransferase might not be involved in its enzymatic activity but in the stability and flexibility of the enzyme in mammalian cells. In addition, according to the current results, the large scale and economic productions of the sialyltransferases including hSAT-I is to be expected using *E. coli*.

Reference

Ishii A *et al* (1998) *J Biol Chem* **273**:31652-31655

C33. Structural glycobiology

C33.1

Glycosylation patterns in Tamm-Horsfall glycoprotein

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Human Tamm-Horsfall glycoprotein (THp) is the most abundant protein in normal human urine, being excreted in quantities up to about 100 mg/day. THp is produced by the kidney where it is expressed via a phosphatidylinositol anchor on the endothelium of the thick ascending limb of the loop of Henle [1]. THp is built up from a polypeptide backbone of 616 amino acids and has a carbohydrate content of 25-30% (w/w) distributed over seven N-glycosylation sites. Detailed structural studies of the N-glycan moiety revealed both oligomannose- type (4 glycans) and complex-type (63 glycans elucidated) structures [2,3]. For the latter type, di-, tri- and, most of all, tetraantennary structures (including dimeric *N*-acetyllactosamine sequences) are present which can be fucosylated, sialylated (including the Sd^a determinant) and/or sulfated. Recently, a FAB-MS study indicated also the presence of core 1 type O-glycans [4].
Here, we focus in more detail on the O-glycan structures using NMR spectroscopy and on the O-glycosylation site using mass spectrometry.

References

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C33.2

Complex neutral N-glycans of *Arion lusitanicus* and *Arion rufus*

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In general there is limited data on the glycosylation of protostomia. We are interested in the glycosylation abilities of gastropods from an evolutionary point of view, and from several aspects of allergy and pest control.
N-glycans of skin, viscera and egg-preparations of *Arion lusitanicus* and *Arion rufus* were released by N-glycanase A. The neutral glycans were separated from the anionic structures by ion-exchange chromatography and the oligomannosidic glycans were removed by digestion with endoglycosidase H. The remaining neutral complex structures were analysed by two-dimensional HPLC and MALDI-TOF mass spectrometry in combination with specific exo-glycosidase digests and by lectin affinity chromatography.
A set of structures was found, ranging from dimethylated $Man_3GlcNAc_2$ carrying β-1,2-linked xylose or α-1,6-linked fucose to larger biantennary xylose and/or fucose containing galactose-terminated structures. Besides a combination of typical animal (e.g. α-1,6-fucose) and plant (e.g. β-1,2-linked xylose) features within the same glycan, a characteristic of gastropod glycosylation seems to be the extended methylation of hexoses (mannose and galactose).

C33.3

Characterization of a novel triphosphonooctaosylceramide from the eggs of the sea hare, *Aplysia kurodai*

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We have isolated a novel glycosphingolipid, named EGL-II, from the eggs of a sea gastropod, *Aplysia kurodai*. By component analysis, sugar analysis, permethylation studies, FAB-MS, SIMS, and ^{1}H-NMR, its structure was revealed to be as follows:

Galα1→3(GlcNAcα1→2)Galα1→3(3-*O*-MeGalα1→2)Galα1→3[6'-*O*-(2-aminoethylphosphonyl)Galα1→2](2-aminoethylphosphonyl →6)Galβ1→4(2-aminoethylphosphonyl→6)Glcβ1→1Cer

The major aliphatic components of the ceramide are palmitic acid, stearic acid, and anteisononadeca-4-sphingenine.

Reference

Araki S *et al* (2001) *J Biochem* **129:**93-100

C33.4

Sialylation of gastropod glycoproteins

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In general there is limited data on the glycosylation of protostomia. We are interested in the glycosylation abilities of gastropods from an evolutionary point of view, and from several aspects of allergy and pest control.
Negative charges on glycans, a common and functionally important feature of higher animals, have been found in protostomia only in a few cases in developmentally young tissues. Previous investigations on N-glycans of *Arionidae*, commonly-occurring slugs causing severe damage in European horticulture, identified the main occurring negative charge on these structures to be *N*-glycolylneuraminic acid.
Here, the investigations are extended to other species (water and land-living snails with or without shells) and the whole spectrum of the glycans in adult tissues (skin and viscera) and eggs. Lectin blots of the extracts, analysis of the enzymatic or acid released sialic acids by HPLC following fluorescent labelling with DMB (1,2-diamino-4,5-methylene-dioxybenzene) and gas-chromatography/mass spectrometry of the trimethylsilylated derivatives revealed a complex picture of sialylation in these organisms.
Sialylation is common in all investigated snails. The amount and type (α-2,3- or α-2,6-linkage) of sialylation depends on species, tissue (skin, viscera) and developmental stage (adult animals, eggs).

C33.5

Glycosyl-phosphatidylinositol (GPI) anchors derived from various vertebrates include β-*N*-acetylglucosaminyl phosphate diester

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GPI anchors appear to be ubiquitous among the eukaryotes and the minimum conserved GPI-core structure found in all GPI-anchors is ethanolamine-PO_4-6Manα1-2Manα1-6Manα1-4GlcN-*myo*-inositol.
Human placental alkaline phosphatase (AP) is reported to be one of the GPI anchor membrane proteins, which has one N-glycan, $(Sia\alpha2\text{-}3)_2$-$Gal_2GlcNAc_2Man_3GlcNAc_2$, and GPI anchor containing ethanolamine phosphate group as a side chain. We found that both asialo-AP and its GPI anchor could bind to a *Psathyrella velutina* lectin (PVL) column, which interacts with β-GlcNAc. PVL binding activity of asialo-AP and its GPI anchor was diminished by digestion with *Diplococcal* β-*N*-acetylglucosaminidase or mild acid treatment. After sequential digestion of AP with β-*N*-acetylglucosaminidase and wheat germ phosphatase, AP showed the respective isoelectric points in accordance with their charges. These results suggested the existence of β-*N*-acetylglucosaminyl phosphate diester attached to GPI anchor of AP. Moreover, we found that these β-*N*-acetylglucosaminyl phosphate diesters are attached to the following GPI anchor membrane proteins; acetylcholine esterases derived from human, bovine, rat and eel, carcinoembryonic antigen, Tamm-Horsfall glycoprotein etc. These results suggested that GPI anchors derived from various vertebrates include β-*N*-acetylglucosaminyl phophate diester.

C33.6

Characterization of novel N-glycans from murine N-CAM

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Polysialylated glycopeptides were isolated from tryptic digests of N-CAM from newborn mouse brain by immunoaffinity chromatography. After desialylation, resulting asialoglycans were tagged with 2-aminopyridine (PA) and separated by HPLC. PA-oligosaccharide fractions obtained comprised neutral as well as negatively charged glycans the latter of which were further analyzed by micromethylation-GLC/MS, MALDI-TOF-MS in conjunction with sequential enzymic degradation and, in part, nano-ESI-MS^n using an ion-trap instrument. Starting from low picomolar amounts of oligosaccharides (10-60 pmol), evidence could be provided that polysialylated murine N-CAM glycans comprise di-, tri- and tetraantennary core structures carrying, in part, type-1 *N*-acetyllactosamine antennae, sulfate groups linked to terminal Gal or subterminal GlcNAc residues and, as a characteristic feature, HSO_3-GlcA-units which are bound exclusively to C3 of terminal Gal in Manα3-linked type-2 antennae. The latter type of glycans represent novel carbohydrate structures. Furthermore, it is remarkable that murine N-CAM carbohydrates may carry within a single glycan polysialic acid and a HSO_3-GlcA-unit which is assumed to represent a HNK1-epitope.

C33.7

Electrospray ionization mass spectrometric analyses of the glycosylation of immunoglobulin G

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Immunoglobulin G (IgG) molecules possess one conserved glycosylation site at Asn-297 in the C_H2 domain of each of the heavy chains, where complex biantennary type oligosaccharides are expressed. The carbohydrate chains exhibit microheterogeneities associated with efficiencies of effector functions and with pathological states. Here, we report mass spectrometric data obtained using a variety of proteolytic fragments of human and mouse IgG. A comparison of electrospray ionization mass spectrometry (ESI-MS) data of Fc fragments that do and do not retain the inter-chain disulfide bridge(s) revealed that (1) the Fc portion can be asymmetric as well as symmetric with respect to glycosylation and (2) the ratios of the individual glycoforms are different from what is expected from the random pairing. It was also revealed that an aglycosyl IgG mutant, in which Asn-297 is replaced by Ala, is *O*-sulfated at Tyr-296.

Reference

Masuda K *et al* (2000) *FEBS Lett* **473:**349-357

C33.8

Human glucuronyltransferase I GlcAT-1: Crystal structures in complex with the active donor UDP-GlcA and acceptor substrates

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GlcAT-I is a α/β globular protein consisting of two sub-domains and appears to form a homodimer with two monomers orienting the substrate-binding cavities in the same surface. The terminal Gal moiety of the acceptor substrate Galβ1-3Galβ1-4Xyl is inserted down into the cavity, while the Xyl moiety resides outside. The active site residues lie in a cleft extending across both subdomains. The terminal Gal interacts with residues, Glu^{227}, Arg^{247}, Asp^{252}, and Glu^{281}. Glu^{281} appears to be a catalytic residue that deprotonates the acceptor group 3OH. The 3OH group is 3.8 Å from the C1 atom of the GlcA molecule of the donor substrate. The GlcA molecule is found interacting with Arg^{156}, Arg^{161}, Asp^{194}, Asp^{252}, and His^{308}. Asp^{194} is a residue within the $D^{194}XD^{196}$ motif that interacts with the Mn^{2+} ion coordinating to the phosphates of the UDP-GlcA. The donor-binding site is conserved in various glycosyltransferases. For bi-functional chondrotin synthase and heparan polymerase, the former enzyme contains two donor-binding sites, while only one such site is evident in the latter. The GlcAT-1 structures provide common basis for the structure and function of glycosyltransferases.

Reference

Pedersen *et al* (2000) *J Biol Chem* **275:**34580-34585

C33.9

Triterpenoid glycosides from *Zygophyllum eichwaldii*

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Twelve triterpenoid compounds were discovered by TLC in the roots of *Zygophyllum eichwaldii* growing in Karakalpakstan. Glycosides C, E and J were isolated by column chromatography from the methanol extract of the plant. On basis of hydrolysis of the glycosides with acid and alkaline solvolysis, and by the data of NMR-spectroscopy with use of COSY, TOCSY, HMQC, HMBC and ROESY methods, it was determined, that compounds C and E are similar with known glycosides: Zyuglycoside II and I isolated from the plant *Sangvisorbia officinalis*, which have the structures 3-*O*-α-L-arabinopyranoside pomolic acid and 28-*O*-β-D-glycopyranosyl ester of 3-*O*-α-L-arabinopyranoside pomolic acid. The new glycoside zygoeichwaloside J is 28-*O*-β-D-glycopyranosyl ester of 3-*O*-β-D-2-*O*-sulfonylgalactopyranoside of pomolic acid.

C33.10

Cycloartane triterpene glycosides from the roots of *Astragalus unifolialatus*

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A novel cycloartane-type glycoside, cyclounifoliozide A, and oleanolic acid were isolated from the roots of *Astragalus unifolialatus Bunge*. Their structures were proved by chemical (alkaline, fermentative hydrolysis) and spectral (IR-, ^1H and ^{13}C NMR) methods. The structure of cyclounifoliozide A was established as 3-*O*-β-D-glucopyranoside; 6,16-di-OAc-24R-cycloartane-3β,6α,16β,24,25-pentaol.
Pharmacological investigations of the methanolic extract of *Astragalus unifolialatus Bunge* showed noticeable hypotenzive action, positive influenced on lipid metabolism.

C33.11

Analysis of *Helicobacter pylori* binding to sialic acid-containing glycosphingolipids

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The human pathogen *H. pylori* is the causative agent for several gastrointestinal diseases such as gastric ulcers and cancer. The bacterium is believed to colonize approximately one-half of the world's human population. It expresses a variety of binding specificities including binding to sialylated glycoconjugates. The aim of our study is to identify the minimum binding structure by investigating the binding of *H. pylori* to modified glycosphingolipids. Sialylparagloboside, known to bind *H. pylori* [1,2], was transformed into its lactone-form by treatment with glacial acetic acid. In another experiment ganglio-tetraosylceramide was derivatized by sialylation by an β-galactoside α-2,3-sialyltransferase (EC number 2.4.99.4). The modifications were confirmed by fast atom bombardment mass spectrometry in the negative mode. The glycolipids were then separated by thin-layer chromatography and overlaid with radiolabeled bacteria. There was no binding of the bacterium to the lactone-form of sialylparagloboside nor to the sialylgangliotetraosyl-ceramide.

References

[1] Miller-Podraza H *et al* (1997) *Infect Immun* **65:**2480-2482
[2] Johansson L *et al* (1998) *Anal Biochem* 2**65:**260-268

C33.12

A model of photoprobe docking with β1,4-galactosyltransferase suggest a carboxylate involved in a SN2 reaction mechanism

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We reported a rapid and efficient method, photoaffinity biotinylation, for identifying photolabeled sites within proteins [1]. Using a photo-reactive biotinyl GlcNAc analog as a nonradioisotopic probe, the approach yielded, for the first time, information on the acceptor site peptides in this enzyme [2]. Here we show a docking model involving the photochemically identified peptide, which suggests a mechanism for the acceleration of galactose transfer. The present model strongly suggests that the carboxylate group of Asp318 could be involved in the activation of the acceptor sugar 4-OH for the efficient galactosyl transfer. The result also exemplified that the combination of photo-affinity labeling with crystallography is a powerful method for the detailed structural analysis of ligand-protein complex [3].

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C33.13

Analysis by mass spectrometry of human leukocyte glycosphingolipids with receptor activity for *Helicobacter pylori* and influenza virus

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Recently we have shown that the human gastric pathogen *Helicobacter pylori* binds to various sialylated glycoconjugates present in human granulocytes [1]. We have also reported a strong binding of human inluenza A to a series of complex NeuAcα6-containing gangliosides isolated from the same source [2]. The nature of the binding structures remains at present unclear. In this work we investigated native and permethylated mixtures of complex glycosphingolipids of human leukocytes using matrix assisted laser desorption/ionization and electrospray/ionization mass spectrometry. The analysis revealed the presence of glycosphingolipids with up to 19 monosaccharides per ceramide. Only neutral and monosialylated glycosphingolipids were clearly seen, however, we could detect fragment ions whose masses agreed with highly sialylated sugar sequences containing up to 4 NeuAcHexHexNAc units. The strong binding of the pathogens in the lower regions of TLC plates indicates that polysialylation/polyvalency of sugar chains may be a crucial factor for binding events *in vivo*.

Reference

[1] Miller-Podraza H *et al* (1999) *Infect Immun* **67:**6309-6313
[2] Miller-Podraza H *et al* (2000) *Glycobiology* **10**:975-982

C33.14

Study of the saccharide component of the carp luteinizing hormone (LH)

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The saccharide component of the gonadotrophic hormone of the carp pituitary was studied. Two α subunits (α_1 and α_2) and one β subunit were obtained by combining different chromatographic procedures, namely chromatography on ConA-Sepharose, gel chromatography and RP-HPLC. The glycoprotein chains were deglycosylated by specific enzymes and their N-terminal amino acid sequence (Edman) and molecular weight (MALDI) were determined. The oligosaccharide chains were derivatized by antranilic acid amide and separated by HPLC on a column of Glyco-SepN using fluorescence detection. The main oligosaccharide fraction of the βLH-subunit was eluted with a retention time corresponding to 7.74 glucose units. This component contains a sulpho group bound via *N*-acetylgalactosamine and galactose to the core of the oligosaccharide. CAMM of carp luteinizing hormone composed of $\alpha_1\beta$ and $\alpha_2\beta$ subunits were constructed. The function of the saccharide chains is discussed.

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C33.15

Carbohydrate structure analyses of zymogen granule membrane glycoprotein GP-2

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GP-2 is the major glycoprotein bound on the inner surface of the zymogen granule membrane (ZGM) by a GPI- anchor. It is thought to play important roles such as sorting of zymogens, creating zymogen granules from the Golgi membranes, and releasing of zymogens. However, the oligosaccharide structures of GP-2 have not yet been thoroughly characterized despite knowledge of the potential N-glycosylation sites on the amino acids sequences. In this study, we analyzed the structures of N-linked oligosaccharides of GP-2.
ZGMs were purified from pancreatic glands of rat, bovine and porcine. Then GP-2 was released from ZGM by PI-specific phospholipase C, and it was characterized by plant lectin blotting and by sugar composition analyses. Furthermore, the structures of pyridylaminated oligosaccharides were studied by 2-D mapping. The results indicated that rat, bovine and porcine GP-2s have similar N-linked oligosaccharides, i.e. triantennary and tetraantennary complex-type oligosaccharides with terminal structures of Neu5Acα2-3Galβ1-4GlcNAc, and the terminal GlcNAc residue substituted with α-Fuc.

C33.16

Structural characterization of type A-active ganglioside variants in type A human erythrocytes

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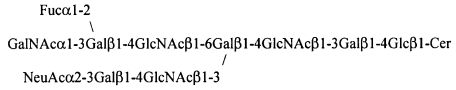
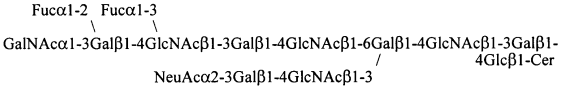
Two monosialogangliosides containing the type A-active epitope have been purified by repeated silica gel chromatography and structurally characterized by methylation analysis by GC/MS, negative SIMS, MALDI-TOF/MS, proton nuclear resonance spectroscopy, and immunological assays, and their structures were concluded to be as follows.

A-active ganglioside I:



A-active ganglioside II:



The reactivity of these gangliosides to the anti-A monoclonal antibodies (mAbs) exhibited enhancement after removal of the sialic acid. Therefore, the sialic acid has been shown to inhibit the binding to the terminal A-active epitope through the formation of an immune complex. The biological significance of these A-active ganglioside variants remains vague at present. As these molecules exhibit different reactivities to the anti-A mAbs, it is likely that they can regulate the antigenicity of the A-epitope on the cell surface.

C33.17

The nematode *Caenorhabditis elegans* synthesises unusual O-linked glycans

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The free-living nematode *Caenorhabditis elegans* appears as a relevant model for studies on the role of glycoconjugates during development of multicellular organisms. Our studies aim to establish the structures of the major O-glycans synthesised by *C. elegans*, in order to give an insight of the endogenous glycosyltransferase activities expressed in this organism. By the use of NMR and mass spectrometry, we have resolved the sequence of two families of O-glycans that present very unusual features. One was characterised by the presence of the GlcA(β1-3)Gal(β1-3)GalNAc-ol sequence, substituted by (β1-4) and (β1-6)-linked Glc residues. The other was represented by a GlcNAc-bound O-glycan substituted by both Fuc and 2-O-Me-Fuc residues. These features contributed to the originality of the glycosylation of *C. elegans*. Our experimental procedure led also to isolate glycosaminoglycan-like components and oligomannosyl type N-glycans. In particular, the data confirmed that *C. elegans* synthesises the ubiquitously found linker sequence GlcA(β1-3)Gal(β1-3)Gal(β1-4)Xyl. Our current efforts tend to isolate the intact mucin-like glycoproteins synthesised by this organism in order to study their individual glycosylation.

C33.18

Age changes in the chondroitin sulphate linkage region of human aggrecan

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The mechanism whereby CS, DS, HS and Heparin are elaborated from a common linkage region is not yet completely established. We have previously shown that the repeat region close to the linkage region of CS from articular cartilage aggrecan has an increased abundance of un- and 4-sulphated GalNAc residues [1,2].
The results of our present study of hexasaccharide linkage regions from human aggrecan of different ages shows an age related increase in both GalNAc 6-sulphation and linkage region Gal sulphation, with a concomitant decrease in unsulphated and 4-sulphated GalNAc residues. There is evidence for only minor changes in these structures after *ca.* 20 years, while there are dramatic changes during development up to this age. Other studies of age related changes in GAG structure have also identified significant changes occurring below this age. (The authors thank the Arthritis Research Campaign (Grant N0521).

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[2] Lauder RM *et al* [2000] *Glycobiology* **10**:393-401

C33.19

Engineering of human sperm membrane antigen HE5/CD52 N-linked glycans

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N-Linked glycans of the sperm membrane antigen HE5/CD52 are candidate contraceptive vaccinogens. The 12 amino acid core peptide carries a unique male-specific carbohydrate epitope which functions as a target for naturally occuring sperm-agglutinating and cytotoxic antibodies involved in antibody-mediated infertility. Male-specific glycoforms of HE5/CD52 containing peripheral α1–3-linked fucose residues are only expressed by the epithelial cells of the distal human epididymis and become inserted into the sperm plasma membrane via a glycosylphosphatidyl-inositol anchor [1]. Our aim is to produce glycoforms of HE5/CD52 in mammalian host cells which can be recognized by the antisperm antibodies and which could form the basis for oligosaccaride-based vaccine development. Glycosylation engineering of BHK and CHO hosts by transfection with genes encoding the terminal glycosyltransferases may allow to obtain such glycoforms. A first step was to analyse the specific inventory of fucosyltransferases in the human epididymal duct epithelium in order to cotransfect the suitable the host cells. Employing real time RT-PCR, all cloned human α1–3-fucosyltransferases were identified in epididymal tissue on the mRNA level, albeit at different quantities.

Reference

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C33.20

NMR structural studies on synthetic modified heparinoid models and their mitogenic activity

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Heparin and heparan sulphate are heterogeneous polysaccharides involved among others processes in several signalling processes the early events of mitogenesis signalling. The signal transmission implies the formation of an extracelular complex between heparin, an extracelular protein, Fibroblast Growth Factors (FGF) and their proteic membrane receptor, Fibroblast Growth Factor Receptors (FGFR). The precise nature of the active FGF-Heparin complex it is not known as contradictory results have been reported depending on the studied system and in the technique used. The main discussion is centred on the role of heparin in the formation of an active FGF dimer. As heparin has a helical structure can present a two equivalent faces and generate FGF dimers under certain conditions.
We have designed and synthesized a hexasaccharide and an octasaccharide where key charged sulphate groups have been removed in one face. Therefore, they should not been able to dimerize FGF. Results on structural studies by NMR and modelling shown that the modification does not change the overall heparinod structure and activity differences should help to discriminate among the proposed interaction models.

Reference

Walksman G *et al* (1998) *Nature Struct Biol* **5:**527-530

C33.21

The 3D structure in solution of the α-subunit of human chorionic gonadotropin

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Human chorionic gonadotropin (hCG) is a glycoprotein hormone produced by the syncytiotrophoblast cells of the placenta. The hormone, built up from two dissimilar non-covalently associated subunits, α and β, contains approximately 30% carbohydrate, and proper glycosylation is thought to be required for biological function. For the determination of the 3D structure of hCG in solution, at first instance attention has been paid to the α-subunit (αhCG), N-glycosylated at Asn52 and Asn78. To this end three probes were investigated by NMR: native αhCG, native αhCG specifically deglycosylated at Asn78, and native αhCG deglycosylated at Asn52 and Asn78, leaving at each site a single GlcNAc residue. As no straightforward strategy was available for the calculation of the NMR structure of intact glycoproteins, a suitable computational protocol, facilitating the successful protein folding in the presence of extended glycans, was developed. The glycans at the two N-sites turned out to be significantly different with respect to their mobility near the Asn residues. Furthermore, the Asn52-containing part of the polypeptide backbone is highly disordered, a phenomenon that does not exist in the crystalline αβ subunits complex. The Asn78-bound GlcNAc shields the protein surface from the hydrophilic environment through interaction with predominantly hydrophobic amino acid residues located in both twisted β-hairpins. Finally, a SPR protocol has been developed to study the heterodimer formation and stability, both in the presence and the absence of the glycan at Asn52.

C34. Tumor glycobiology

C34.1

Selective expression of gangliosides 3'-isoLM1 and GD3 in proliferating and migrating tumor cells

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Aberrant ganglioside expression in the brain tumor malignant glioma, compared to normal brain has been shown earlier by biochemical analyses. In this study the in vivo expression and distribution of the glioma-associated gangliosides, GD3 and 3'-isoLM1 (NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-Cer) was investigated by immunohistochemistry and confocal microscopy. A novel human brain tumor nude rat xenograft model in which the tumors, like in patients, show extensive infiltrative growth within the rat brain, was used. The results showed that ganglioside GD3 was expressed in the tumor parenchyma, dominated by proliferating glioma cells, while ganglioside 3'-isoLM1 was more abundantly expressed in the periphery of the tumor, associated with areas of tumor cell invasion. Moreover, double staining with a pan anti-human monoclonal antibody (3B4) and the anti-ganglioside monoclonal antibodies confirmed that the ganglioside expression was associated with tumor cells. This supports the concept of different biological roles for individual gangliosides and that gangliosides GD3 is involved in cell proliferation and 3'-isoLM1 possibly in processes associated with glioma cell migration.

C34.2

Regulation of Lewis antigens on human hepatocarcinoma cell line and its relation to metastatic potential *ex vivo*

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Human hepatocarcinoma cell line 7721 expressed mainly sialyl Lewis X (SLe^x) and low level of sialyl dimeric Lewis X ($SDLe^x$) antigens on the cell surfaces, but only a trace amount of Lewis X (Le^x) and sialyl Lewis A (SLe^a). The expressions of SLe^x and $SDLe^x$ were downregulated by two differentiation-inducers, all-*trans* retinoic acid (ATRA), 8-bromo-cyclic AMP (8-Br-cAMP); and upregulated by three proliferation-inducers, epidermal growth factor (EGF), phobol-12-myristate-13-acetate (PMA) and insulin. Transfection of *c-erb*B2/*neu* and *nm*23-H1 gene into 7721 cells also increased and decreased the expression of SLe^x, respectively. The expressions of metastasis-related phenotypes, cell adhesion to laminin (Ln) or human umbilical vein endothelial cells (HUVEC), cell migration through transwell and invasion through matrigel, were changed in the same direction and in proportional with the expression of SLe^x. The specific monoclonal antibody (mAb) of SLe^x (KM93) significantly, and mAb of $SDLe^x$ (FH6) slightly, but antibodies of Le^x and SLe^a did not abolish the cell adhesion to HUVEC, cell migration and invasion. Transfection of α1,3FuT-VII cDNA into 7721 cells showed similar results as the treatment of proliferation-inducers or the transfection of *c-erb*B2/*neu*. The above results indicated that there was a close relationship among the expressions of SLe^x and the metastatic potential of hepatocarcinoma cells. (Supported by grant from NSFC 39630080).

C34.3

Proteoglycans as targets for antitumour therapy

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Proteoglycans (PG) are known to participate in the regulation of growth and angiogenesis. We have studied the biosynthesis and turn-over of glypican-1 in transformed cells. There is extensive internalization, degradation of the heparan sulphate (HS) side-chains, recycling of the core protein and resynthesis of HS. Degradation is caused by endoheparanase and by NO-derived nitrite that cleaves at occasional $GlcNH_3^+$. Glypican participates in the uptake of polyamines when endogenous polyamine synthesis is inhibited. Polyamine-deprivation results in an increased content of $GlcNH_3^+$ in HS. Polyamine uptake is inhibited in nitrite-deprived cells. Recycling glypican does not enter the nucleus but free HS chains are transported to the nucleus. PG-deficient cells are growth-stimulated by exogenous HS when made dependent on polyamine uptake. Furthermore, results of experiments using an animal tumour-model show that PG-deficiency renders tumour cells more sensitive to polyamine synthesis inhibition. As xylose occupies the unique position as the link between HS chains and the core protein, xylosides can be used to manipulate PG synthesis. Xylosides with specific aglycones inhibit selectively growth of transformed cells.

Reference

Fransson L-Å *et al* (2000) *Matrix Biol* **19:**367-376

C34.4

Carboxymethylated glucans as macrophage stimulators and antitumor effect in combination with cyclophosphane

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Water-insoluble (1-3)-β-D-glucans from the cell walls are effective stimulators of the host immune system, however, poor solubility limited their usage in clinical medicine. In order to improve the solubility of β-glucans, carboxymethylation was suggested. The aim: to evaluate the effect of these glucans on tumor growth. Carboxymethylated (1-3)-β-D-glucans (CMG) from *Saccharomyces cerevisiae* (DS 0.7-0.9 - for the 2^{nd} fraction) were administered i.p. to CBA/C57Bl/6 mice in a dose of 25 mg/kg body weight. The combined treatment by cyclophosphane (CPA, 150 mg/kg) and CMG, 2^{nd} fraction, revealed a significant inhibition of the growth of the intramuscular transplantant of Lewis adenocarcinoma (90%) and its metastasizing to lung (90%). CPA alone inhibited tumor growth (45%) and metastasizing (58%); CMG alone only decreased the number of metastases (70%). So, the most significant tumor growth and metastasizing inhibition was shown in combined treatment by CPA and CMG. So, CMG increased the host resistance and tumoricidal activity of macrophages during combined treatment with CPA. Non-toxic CMG 2^{nd} fraction is perspective for therapy of tumors and prophylaxis of metastasizing.

Reference

Korolenko T *et al* *Proc. 3rd World Meeting APV/APGI, Berlin*, 3/6 April 2000, pp 343-344

C34.5

Candidate molecules for leczyme receptor on leukemia P388 cells

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Leczyme from *Rana catesbeiana* eggs is a sialic acid-binding lectin (SBL) with intrinsic RNase activity and it induces apoptosis in mouse leukemia P388 cells. We have demonstrated that leczyme receptor (SBLR) was detected in the Triton-insoluble (TI) fraction of various tumor cells by SDS-PAGE, followed by Western blotting with SBL and anti-SBL antibody. The apoptotic effect of SBL decreased in phosphatidylinositol-specific phospholipase C (PI-PLC)-treated P388 cells. Sialic acid content of PI-PLC-treated cells was reduced to about one-half non-treated cells. These results suggest that candidate molecules for SBLR are GPI-anchored glycoproteins having sialic acid residues. SBLR high-expressed ($SBLR^H$) and low-expressed cells were separated from parent P388 cells by the use of magnetic cell sorting (MACS) system (Vario MACS, Miltenyi Biotec) with goat anti-rabbit IgG MicroBeads. SBLRs with molecular weights between 115 and 150 kDa were detected in the TI fraction from $SBLR^H$ cells. The reaction medium obtained by treatment of $SBLR^H$ cells with PI-PLC was separated by the use of μMACS with protein G MicroBeads. This PI-PLC treatment resulted in releasing the bands of a membrane-bound form described above, as a soluble form. Binding of SBL to GPI-anchored glycoproteins raises an interesting question regarding the correlation of SBLR with Fas antigen- or TNF receptor-mediated signaling in membrane rafts.

C34.6

Elevated level of syndecan-2 expression suppresses the metastatic phenotype of Lewis lung carcinoma cells

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A Lewis lung carcinoma-derived low metastatic P29 clone exhibits tumorigenesis dependent on the fibronectin (FN)–rich stromal matrix, whereas a highly metastatic LM66-H11 clone shows basement membrane-dependent tumorigenesis. This difference was reflected in actin cytoskeletal organizations on a FN substratum *in vitro*; P29 cells formed stress fibers, whereas LM66-H11 cells formed cortex actin. The stress fiber formation in P29 cells was shown to be induced by cooperative signaling through integrinα5β1 and syndecan-2 and inhibited by treatment with its antisense oligonucleotide of the cells, causing the localization of actin filaments in membrane periphery of the cells to form cortex actin. The latter phenotype was indistinguishable from that of LM66-H11 cells. These results led us to investigate the biological functions of syndecan-2. After transfection of syndecan-2 cDNA into the highly metastatic LM66-H11 cells, a stable transfectant expressing syndecan-2 at a level similar to that of the P29 clone was established. This transfectant resembled the P29 clone in the important cellular characteristics including cell morphology, doubling time, saturation density, and the actin cytoskeletal organization formed on adhesion to the FN substratum. Moreover, when injected intravenously and subcutaneously, the transfectant showed surprisingly a marked decrease in pulmonary metastasis to a degree that the P29 clone showed. We finally found that there was an inverse correlation between the cell surface expression of syndecan-2 and metastasis in Lewis lung carcinoma.

C34.7

Overexpression of sialyltransferase ST6GalNAc-II is related to poor patient survival in human colorectal carcinomas

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The Thomsen-Friedenreich antigen (TF) belongs to a family of tumor-associated carbohydrates. Expression of TF on glycoconjugates has been correlated with tumor prognosis and metastasis. The aim of the present study was to examine tumor-associated alterations of glycosyltransferases involved in the biosynthesis of the TF-glycotope in colorectal carcinomas. To this end, expression of sialyltransferases ST3Gal-I, ST3Gal-II, ST6GalNAc-II and of core 2 ß1,6-*N*-acetylglucosaminyltransferase was examined by RT-PCR in forty cases of colorectal carcinoma specimens and in "normal" mucosa of the same patients. Occurrence of TF was detected by immunohistochemistry using the monoclonal antibody A78-G/A7 only in 7% of the normal mucosa, but in 57% of the carcinoma samples. Expression of α2,3-sialyltransferases ST3Gal-I, ST3Gal-II and α2,3-sialyltransferase enzyme activity was significantly increased ($p<0.001$) in carcinoma specimens. ST6GalNAc-II mRNA expression was significantly increased ($p=0.04$) in cases with metastases to lymph nodes along the vascular trunk. Moreover, ST6GalNAc-II expression provides an prognostic factor for patient survival (log rank, $p=0.02$). In an attempt to study the functional relevance of the glycosyltransferases for TF-biosynthesis, SW480 colorectal cells were transfected with each of the enzymes. The presence of TF was not altered by transfection of the cells with either sialyltransferase ST3Gal-I or ST3Gal-II. However, successful transfection with C2GNT led to reduced expression of TF. In contrast, increased cell surface expression of TF was found after ST6GalNAc-II transfection. Thus, the expression of TF on the cell surface of SW480 colorectal carcinoma cells depends on the ratio of C2GNT and ST6GalNAc-II.

C34.8

Synthetic disaccharide primers inhibit sialyl Lewis X-mediated tumor cell adhesion

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The cell surface carbohydrate antigen, sialyl Lewis X (sLe^X), is expressed on many carcinomas and facilitates tumor metastasis by binding to the selectin class of cell adhesion receptors located on platelets and endothelia. Patient survival studies after surgical resection of tumors indicate higher mortality for those tumors expressing sLe^x compared to those that do not express those antigens. Therefore, inhibiting sLe^X on tumor cells may improve patient survival, by blocking adhesion and formation of thromboemboli. Synthetic disaccharides, acetylated Galβ1-4GlcNAcβ-O-naphthalenemethanol (NM) and GlcNAcβ1-3Galβ-O-NM, were found to act as "primers" of oligosaccharide synthesis, generating products related to mucin-like chains related to Lewis antigens. Priming diverts the assembly of the chains from endogenous glycoproteins resulting in inhibition of selectin-dependent tumor cell adhesion in cultured U937 cells. We have now extended these findings to carcinoma cells, demonstrating the utility of the disaccharides as antagonists of tumor-cell adhesion of selectins mediated by sLe^X. Tumor cells treated with the compounds causes a reduction in their adhesion to purified selectins, activated endothelial cells, and platelets. It also alters survival of the cells in an experimental mouse metastasis model and a >3 fold reduction in pulmonary metastasis when compared with control mice. This novel class of synthetic disaccharides can therefore potently inhibit selectin-mediated adhesion of colon and lung tumor cells and block tumor formation.

C34.9

Resistance of β1,4-*N*-acetylglucosaminyltransferase III transgenic mice to hepatocarcinogenesis by diethylnitrosamine

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The β1,4-*N*-acetylglucosaminyltransferase III (GnT-III) is involved in the formation of branches in asparagine-linked oligosaccharides. While expression of GnT-III in the liver is quite low, it is increased during hepatocarcinogenesis. Until recently, the biological significance of this up-regulation has remained obscure. In the present study, the biological effect of GnT-III on tumor formation in the liver was investigated, using GnT-III transgenic mice. When these mice were treated with low dose of diethylnitrosamine (DEN) via intraperitoneal injection, the incidence of hepatic tumor was dramatically suppressed in the GnT-III transgenic mice (0.05 ± 0.23 in GnT-III transgenic mice *vs.* 1.38 ± 1.78 in control mice, *mean* ± SD, $p < 0.01$). Administration of a high dose of DEN led to tumor formation in both GnT-III transgenic and control mice (2.33 ± 3.94 in GnT-III transgenic mice *vs.* 3.60 ± 5.70 in control mice, *mean* ± SD). Two-dimensional electrophoresis followed by lectin blot analysis revealed that haptoglobin was strongly glycosylated in the hepatic tumor-bearing GnT-III transgenic mice. These results imply that the ectopic expression of GnT-III in the mice tends to suppress chemical carcinogenesis and haptoglobin is one of the target molecules of GnT-III during hepatocarcinogenesis.

C34.10

GlcNAc octaclusters activate natural killer cells through NKR-P1 and are promising antitumor compounds

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Glyodendrimers are synthetic glycoconjugates (neoglycoconjugates) with interesting biomedical applications. We have found that GlcNAc octaclustered through a polyamidoamine linkage (prepared for us by TK Lindhorst, University of Kiel, Germany), is a good ligand for the activating receptor of rodent natural killer cells, NKR-P1. GlcNAc dendrimers were thus evaluated as antitumor compounds. They were shown to be able to attract $NKR\text{-}P1^+$ cells to the sites of tumors, and activate the local immunity (cytotoxicity of lymphocytes).

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C34.11

Expression of CD77/Gb3 synthase gene in human hematopoietic cells

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CD77/Gb3 is a Burkitt lymphoma associated glycolipid antigen, and also a receptor for O157 *E. coli* Shiga-like toxin (verotoxin). Physiological roles of CD77/Gb3 has not been well understood. We have analyzed the expression levels of CD77/Gb3 synthase (α1,4-galactosyltransferase) gene in human hematopoietic cells and cell lines together with those of CD77 expression. The expression levels of the synthase gene well correlated with those of the antigen itself. In addition to Burkitt lymphoma cells, megakaryocytic leukemia cell lines also expressed CD77/Gb3 synthase gene. Then, we analyzed the effects of TPA-induced differentiation of MEG-O1 (a megakaryocytic leukemia line) on the CD77/Gb3 expression. Although surface expression of the antigen reduced, mRNA level of CD77/Gb3 synthase gene rather increased. Therefore, we analyzed the intracellular localization of CD77/Gb3 during the induced differentiation in MEG-O1 cells. CD77/Gb3 was strongly stained as granules in the cytoplasm after differentiation. There were also a lot of granular particles stained around MEG-O1 cells. Some of these granules were also stained by anti-GPIIb-IIIa antibody, suggesting that CD77/Gb3 is transferred from surface membrane to platelets under generation as found granular particles in the cytoplasm and around cells.

C34.12

Insights into new biological functions of tumor gangliosides

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We present new evidence implicating tumor gangliosides in enhancing tumor formation by influencing the interaction between tumor cells and their microenvironment: (a) In a new syngeneic murine lymphoma model, shed tumor gangliosides inhibited the tumor specific primary and secondary proliferative responses, the generation of tumor-specific cytotoxic lymphocytes, and the immune response to tumor cell challenge *in vivo*. (b) Alteration of ganglioside metabolism by inhibition of glucosylceramide synthase, either pharmacologically or by antisense transfection (which did not alter cell morphology and proliferation kinetics), caused 50-90% reduction in tumor formation and metastatic capability of murine melanoma cells. (c) Membrane ganglioside enrichment enhanced EGF-induced proliferation of human fibroblasts and VEGF-induced proliferation and migration of human vascular endothelial cells, as well autophosphorylation of the VEGF receptor and activation of Ras and MAP kinase in fibroblasts. These findings, showing that tumor gangliosides may alter multiple tumor host interactions to favor tumor progression, suggest a new therapeutic approach to human cancer.

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C34.13

Decreased cell proliferation and motility of mammary carcinoma cells by sialyltransferase ST6GalNAc I gene transfection

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Sialyl-Tn antigen (STn) is strongly expressed in carcinomas but it is unclear if it affects cells' behavior. STn corresponds to the disaccharide NeuAcα2,6GalNAc. Its synthesis requires an ST6GalNAc transferase. Addition of the α2,6-linked sialic acid blocks elongation of the O-glycan chains. To generate STn epitopes and to block O-glycans elongation in tumor cells, we transfected a mouse breast carcinoma cell line (TS/A) with the human ST6GalNAc I cDNA. After cloning, STn+ cells were obtained. They showed a dramatic decrease of reactivity with the peanut and jacalin lectins indicating that addition of the α2,6-linked sialic acid did block synthesis of core 1 O-glycans. As compared with controls transfected with empty vector, STn+ transfectants showed reduction of both proliferation and motility on fibronectin. However, cell movement on collagens was not altered. In an *in vitro* wound healing assay, STn+ cells showed lower repair speed. They presented an elongated morphology with a clear impairment of actin stress fibers' organisation. Upon s.c. injection to syngeneic mice, tumors from STn+ cells grew as rapidly as tumors originating from control cells. However, antigenic expression was lost *in vivo*, indicating that the growing tumor cells were loss variants. Thus in the TS/A model, blocking O-glycans' elongation by ST6GalNAc I transfection severely affects cellular morphology, proliferation, motility and tumorigenicity.

C34.14

Clinical significance of ST3Gal IV expression in human renal cell carcinoma

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Sialyltransferase expression has been reported to be altered in carcinogenesis. Out of sialyltransferases cloned to date, we focused on ST3Gal IV expression in human renal cell carcinoma (RCC). Levels of ST3Gal IV mRNA were examined in human RCC in comparison with non-tumor kidney. ST3Gal IV cDNA obtained by PCR from cDNA library of human RCC cell line ACHN was identical to STZ in nucleotide sequence. Northern blot analysis was performed for 24 non-tumor kidney and primary RCC tissues, and 4 metastases. ST3Gal IV mRNA level was decreased in 16cases of 23 primary RCC tissues compared to 20 non-tumor kidney tissues. The mRNA level was low in 3 and equivocal in one, of 4 metastases. The 6 cases of RCC tissues that possessed relatively high ST3Gal IV mRNA level showed favorable prognoses. These results suggest that down-regulation of ST3Gal IV mRNA may be associated with malignant progression of human RCC.

Reference

Kitagawa H and Paulson JC (1994) *J Biol Chem* **269**:1394-1401

C34.15

Remodeling of N-linked oligosaccharides of tissue inhibitor of metalloproteinase-1 (TIMP-1) by β1,4-*N*-acetylglucosaminyl-transferase-III results in increase of inhibitory activity against matrix metalloproteinase-9 (MMP-9)

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It is a well-known fact that N-linked oligosaccharides of glycoprotein control biological character of tumor cells. We previously reported a suppression of lung metastasis of B16 mouse melanoma cells by N-acetylglucosaminyltransferase-III (GnT-III) gene transfection (*PNAS*: 92:8754-8758, 1995). One of the mechanisms was found to be a decrease of the invasive ability via cadherin in the GnT-III transfected cells. Many other glycoproteins such as proteases are involved in the tumor invasion. In the present study, effects of remodeling of N-linked oligosaccharides by GnT-III were investigated in terms of the matrix metallopreoteinases (MMPs) / tissue inhibitor of metalloproteinases (TIMPs) system. Although no difference in the expression level of MMPs was observed between the GnT-III transfectants and control cells, the enzymatic activity of MMPs in the conditioned medium from the GnT-III transfectants was decreased. TIMP-1 purified from the GnT-III transfectants was smaller than that from control cells and its binding to E4-PHA lectin was increased, suggesting that N-linked oligosaccharide of TIMP-1 was modified by GnT-III. Inhibitory activity against MMP-9 of the purified TIMP-1 derived from the GnT-III transfectants was slightly increased as compared to that from control cells. These findings suggest that modification of TIMP-1 function by GnT-III is involved in the decrease in tumor invasion of GnT-III transfectants. This is the first report that changes of glycoprotein function by GnT-III were directly shown at a single protein level.

C34.16

E-cadherin function modulated by GalNAcα-OBn on HT 29 cells

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We previously showed that permanent exposure of the human colon cancer cell line HT 29 to the sugar analogue GalNAcα-OBn (BgN) alters the intracellular trafficking of some glycoproteins. We sought to define the effect of BgN on statement and function of E-cadherin, the main cell-cell adhesion molecule, in three HT 29 cell lines, HT 29 standard, HT 29-5M21 and HT 29-5F7, the former being the parental population and the two latter being clones of cells differentiated into a mucin-secreting and an enterocytic phenotype respectively [1]. We first studied effect of BgN on statement of E-cadherin. We then sought to define functional consequences on the aggregation and invasion properties of the cells on collagen type I. HT 29 standard were non invasive and showed aggregation that depends on E-cadherin. The aggregation decreased with BgN, leading to an increased invasiveness. In contrast, HT 29-5M21 and HT 29-5F7 were invasive, with an E-cadherin independent aggregation for HT 29-5M21 and absence of aggregation for HT29-5F7. BgN decreased the invasiveness and induced the aggregation in HT 29-5M21 on a E-cadherin dependent way. In contrast, there was no effect of BgN on HT 29-5F7 behavior.

Reference

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C34.17

The roles of P-selectin, L-selectin and carcinoma mucins in Trousseau's Syndrome

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Trousseau's syndrome is defined as spontaneous, migratory thrombophlebitis associated with an underlying, often occult, cancer. The prevailing hypothesis is that this syndrome is caused by the release of tumor-associated tissue factor into the blood, thereby activating the extrinsic pathway of blood coagulation. Trousseau's syndrome is often associated with mucin-producing carcinomas, and mucins are known to circulate in the blood of many cancer patients, often being used as prognostic markers. The aim of our work is to determine if circulating mucins that have binding sites for the selectin family of adhesion molecules can contribute to the pathophysiology of Trousseau's syndrome. Highly purified carcinoma mucins (characterized by chemical composition, and free of tissue factor or pyrogens) are intravenously injected into either wild-type or selectin-deficient mice. Preliminary histological and hematologic analyses of such mice indicate that these mucins can initiate platelet aggregation and thrombosis in a manner dependent on P-selectin, and possibly L-selectin.

C34.18

Suppressed expression of α1,2-fucosyl residues on colorectal carcinoma cells was accompanied by decreased tumorigenicity

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The accumulation of α1,2-fucosylated antigens such as Le^b, Y and H type II has been observed in human colorectal carcinoma cells and its enzymatic analysis has also been reported in our previous studies [1-3]. In this study, we investigated mouse colon26 cells transfected with the genes encoding H and Le enzyme to show the acquisition of both the expression of aforementioned fucosylated antigens on the cell surface and the resistance to anti-cancer treatments with 5-fluorouracil and UV. A series of human colorectal carcinoma cell lines whose cell surface expressed much fucosylated antigens were also examined and found to possess resistance to such treatments. Conversely, the suppressed expression of α1,2-fucosyl residues on their cell surface was demonstrated to increase the susceptibility to those treatments. Further, it was suggested that the H enzyme acceptors could be used as an inhibitor to block the expression of α1,2-fucosyl residues on the cells resulting in the decrease of their tumorigenicity.

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C34.19

Selective antiproliferative action of 2-(6-hydroxynaphthyl)-*O*-β-D-xylopyranoside (Xyl-2-Nap-6-OH) - mechanistic studies

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Different xylosides have been synthesized and tested for growth inhibition and priming of heparan sulphate (HS) in normal and transformed cells. Selective growth-inhibition by Xyl-2-Nap-6-OH is dependent on 6-OH. Xylosides without OH, with 6-OMe or with 7-OH are all inactive. Free aglycones are sometimes more toxic than the corresponding xyloside excluding hydrolysis as a mechanism. Ability to prime HS synthesis is not unique to Xyl-2-Nap-6-OH. Mutant CHO cells unable to synthesize HS are insensitive to the xyloside, whereas wild-type cells are inhibited. Thus, HS synthesis is a necessary but insufficient requirement. Xyloside-primed HS chains contain $GlcNH_2$ residues susceptible to degradation by NO-derived nitrite. Growth-inhibition by Xyl-2-Nap-6-OH is abrogated in nitrite-deprived cells. We propose that Xyl-2-Nap-6-OH is a pro-drug that primes HS synthesis. The secreted chains are taken up and degraded to the active compounds consisting of a HS-stub linked to a possibly modified aglycone.

Reference

Mani K *et al* (1998) *Cancer Res* **58:**1099-1104

C34.20

Preliminary characterisation of cadherin glycans from human urinary bladder carcinoma cell line T-24

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Cadherins are a family of transmembrane glycoproteins involved in intercellular adhesion. It has been shown that aberrant glycosylation of E-cadherin contributes to the suppression of metastasis in murine melanoma cells. Cadherin from cultured T-24 line of human bladder carcinoma was precipitated using the Pan-Cadherin mAb and purified by SDS-PAGE, followed by Western blotting on Immobilon P. Carbohydrate moieties of N-cadherin were released on-blot deglycosylation using PNGase F. Profiles of natural neutral N-glycans obtained by matrix-assisted laser desorption/ionisation mass spectrometry (MALDI MS) revealed the presence of high mannose with and without core fucose as well as hybrid type rich in fucose residues.

C34.21

Suppression of tumor growth by transfection of human β-1,4-galactosyltransferase (β-1,4-GalT) gene into B16-F10 mouse melanoma cells

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Upon malignant transformation of cells, activities of *N*-acetylglucosaminyltransferase V which is involved in formation of highly branched N-linked oligosaccharides have been shown to increase by 4-10 folds. However, no significant change in β-1,4-GalT activities was found in tumors and cancer cells. Investigation of the gene expression of the β-1,4-GalT family members in a variety of cancer cells and their normal counterparts available revealed that the gene expression of β-1,4-GalT II decreases and that of β-1,4-GalT V increases without altering others by malignant transformation. To examine whether such changes are essential for malignancy of cancer cells, human β-1,4-GalT II sense or V antisense cDNA was introduced into B16-F10 mouse melanoma cells to revert the galactosylation, and the cells were transplanted subcutaneously into C57B/L6 mouse. The mock-transfectants developed tumors as big as 13 mm in two weeks while both gene-transfected cells formed tumors with 4 mm wide in the same period. Lectin blot analysis of membrane glycoprotein samples from these tumors showed significant changes in RCA-I binding among samples. The results indicate that the changes in the gene expression levels of β-1,4-GalTs II and V are essential for tumor growth.

C34.22

Structural characterization of N-cadherin glycans from human melanoma cell lines

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The expression of N-cadherin in tumor cells is associated with their invasive potential [1]. Many studies suggested the contribution of N-linked oligosaccharides of proteins in cancer metastasis [2]. We analysed the carbohydrate profile of N-cadherin glycans obtained from human melanoma cell lines; WM35 from primary tumor site and WM239, WM9, A375 from metastatic sites. Lectin analysis of immunoprecipitated N-cadherin indicated the differences between the N-cadherin glycans from primary tumor (WM35) and from metastatic sites (WM239, WM9 and A375). N-Cadherin from WM35 contains high-mannose type and complex biantennary glycans with α2-6 linked sialic acid. N-cadherin from all metastatic lines possesses tri- or tetra-antennary complex type glycan with α-fucose. N-Cadherin from WM9 (lymph node metastatic line) and A375 (solid tumor metastatic line) contains complex type chains with sialic acid. Preliminary MALDI MS analysis of oligosaccharides released from immuno-precipitated N-cadherin (A375 cell line) confirmed the presence of tri- or tetra-antennary complex type glycan with α-fucose.

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C34.23

Addition of bisecting GlcNAc residues to E-cadherin downregulates the tyrosine phosphorylation of β-catenin

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The enzyme GnT-III (β1,4*N*-acetylglucosaminyltransferase III) catalyzes the addition of a bisecting *N*-acetylglucosamine (GlcNAc) residue on glycoproteins. Our previous study described that the transfection of GnT-lll into mouse melanoma cells results in the enhanced expression of E-cadherin, which, in turn, leads to the suppression of lung metastasis. It has recently been proposed that the phosphorylation of a tyrosine residue of β-catenin is associated with cell migration. The present study reports on the importance of bisecting GlcNAc residues on tyrosine phosphorylation of β-catenin. An addition of bisecting GlcNAc resi-dues to E-cadherin leads to an alteration in cell morphology and the localization of β-catenin after EGF stimulation. These changes are the result of a downregulation in the tyrosine phosphorylation of β-catenin. In addition, tyrosine phosphorylation of β-catenin by transfection of constitutively active c-src was suppressed in GnT-III. Thus, the addi-tion of the bisecting GlcNAc to E-cadherin-β-catenin complex down-regulates the intracellular signaling pathway, suggesting its implication in cell motility and the suppression of cancer metastasis.

Reference

Kitada T *et al* (2001) *J Biol Chem* **276:**475-480

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