GLYCO XVIII

XVIII International Symposium on Glycoconjugates

Abstracts

September 4-9, 2005
Firenze, Italy

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PLENARY LECTURES

PL01
FROM MICROMOLES TO FEMTOMOLES OF GLYCOPOLYMERS: A QUARTER OF A CENTURY OF MS STRUCTURE ANALYSIS

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In the glycobiology field no structural technique can match mass spectrometry for the breadth of problems that can be addressed, the complexity of samples that can be successfully analysed and for the amount of structural information that can be obtained from tiny quantities of material. The introduction of high field mass spectrometers in the late 1970s, followed a few years later by fast atom bombardment ionisation (FAB-MS), revolutionised the structural characterisation of many glycopolymers. A decade later, a second revolution occurred when two new ionisation methods of exceptional sensitivity were introduced, namely Matrix Assisted Laser Desorption Ionisation (MALDI) and Electrospray Ionisation (ESI). In the 1990's major advances were made in tandem instrumentation, for example the introduction of the quadrupole orthogonal acceleration time of flight analyser (Q-TOF), resulting in unprecedented capabilities for ultra-high sensitivity sequencing.
In this lecture I will present some of the glycobiological highlights of the MS-based structural studies that have been the focus of my laboratory during the past twenty five years. The current capabilities of MS technologies will be exemplified by investigations of the glycan repertoire of a variety of glycopolymers, cells, tissues, secretions etc from a range of sources.

PL02
THE EMERGING ROLE OF CARBOHYDRATES IN T CELL RECOGNITION, IMMUNOLOGICAL TOLERANCE AND AUTOIMMUNE DISEASES

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The immune system has evolved elaborate mechanisms to repel a myriad of microbial and viral invaders encountered throughout life. This is achieved by the ability of lymphocytes to recognize $>10^8$ different antigens, yet at the same time be tolerant towards self-antigens. Traditionally T lymphocytes have been considered to recognize exclusively peptide antigens complexed to MHC molecules. However, recent work has shown that carbohydrates can play major roles in influencing antigen processing, MHC binding of epitopes and T cell recognition. The initiation of autoimmunity occurs when tolerance to self-antigens is broken. Many potential targets for pathogenic self-reactive T cells are glycoproteins. It is clear that T cell tolerance to self-antigens depends on many factors including the precise levels of antigen dose. Therefore, alteration of the glycosylation of self-antigens has the potential to influence the level of epitope production, or the generation of neoepitopes, leading to autoimmune responses. The lecture will review the potential roles of carbohydrates in the biology of T cell responses and highlight work in the author's laboratory on a highly defined model of an organ-specific autoimmune disease, namely autoimmune gastritis. In this autoimmune disease the principal self-antigen that is recognized by pathogenic T lymphocytes is the highly glycosylated β-subunit of the gastric H/K ATPase. Our current understanding of the mechanisms of T cell tolerance to this gastric glyco-antigen, and the loss of self-tolerance leading to autoimmunity, provides the basis for defining the role of glycosylation in these complex pathways.

PL03
TWENTY-ONE YEARS OF O-GLCNAC: EMERGING ROLES IN SIGNALING, TRANSCRIPTION, STRESS AND PROTEIN TURNOVER.

Gerald W. Hart, Natasha Zachara, Chad Slawson, Kaoru Sakabe, Win Cheung, Stephen Whelan, Pui Butkinaree, Mike Housley, Kyoungsook Park, Shino Shimoji, Zihao Wang, Quira Zeidan, and T. Lakshmanan.
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It has been over 21-years since nucleocytoplasmic protein glycosylation by O-GlcNAc was reported (*JBC 259, 3308-3317)*. During these past two decades, we have continued to be astonished by the abundance, the distribution on many different proteins, the dynamics, the biological complexity and the structural diversity caused by the cycling of this monosaccharide on regulatory proteins. It is now clear that O-GlcNAcylation serves as a "metabolic sensor" that modulates signaling, transcription, and other cellular processes in a global fashion in all metazoans. O-GlcNAc also plays a key role in human disease, including diabetes, heart disease, age related neurodegeneration, and cancer. The molecular functions of this dynamic saccharide include: regulation of phosphorylation, modulation of protein:protein interactions, protein trafficking, and blocking protein degradation. The dynamic interplay between O-GlcNAc and O-phosphate, the roles of the saccharide in the transcription machinery, and its roles in glucose toxicity are key focus areas. Our understanding of the molecular mechanisms regulating almost every key cellular process will require elucidation of the roles of O-GlcNAcylation, as well as understanding the roles of other post-translational modifications.

Supported by NIH grants HD13563, CA42486, DK61671, DK71280, and NIH contract N01-HV-28180. Dr. Hart receives a share of royalty received by the university on sales of the CTD 110.6 antibody. The terms of this arrangement are being managed by The Johns Hopkins University in accordance with its conflict of interest policies.

PL04
A GLYCOBIOLOGICAL VIEW OF FERTILIZATION

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It is an essential requisite for fertilization that spermatozoa recognize and react properly to the glyco-signals embedded in the egg coats. An overview of the present knowledge on the structures, functions and receptors of the glycol-signals will be presented and discussed in reference to speciation.

PL05
COMPREHENSIVE STUDY ON HUMAN GLYCOSYLTRANSFERASES, ENZYMATIC SYNTHESIS OF GLYCAN LIBRARY, AND HIGH THROUGHPUT ANALYSIS OF GLYCAN STRUCTURE USING MS^N WHICH ARE DEVELOPING A DATABASE FOR GLYCOMICS.

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For these four years, we performed a comprehensive study on human glycosyltransferases with the aid of bioinformatic technology. One hundred five novel glycogens, including glycosyltransferases, sulfotransferases and sugar-nucleotide transporters, were identified as candidates. All of them were cloned and expressed as recombinant enzymes, and their substrate specificities were then examined using various acceptors. At the present time, one hundred sixty-five gylcogenes were subcloned into a Gateway entry vector, and prepared as a human glycogene library. These cloned glycogenes can be easily expressed as recombinant enzymes in various expression systems as active enzymes [1)]. Next, we developed a construction method for a molecular weight tagged library for the construction of oligosaccharide and glycopeptide libraries using the above glycosyltransferase library. The libraries were useful for rapid and easy screening not only for ligand specificity of carbohydrate-binding proteins such as lectins and antibodies but also for binding specificity of virus and bacteria [2)]. Using these standard oligosaccharide libraries, we are developing a technology for the rapid and accurate identification of the oligosaccharide structures on glycoproteins using only mass spectrometry [3)]. Finally, we are constructing a carbohydrate sequencing database named "CabosDB". CabosDB consists of glycogene DB, oligosaccharide DB, lectin DB and glycoprotein DB [4)]. The details of Cabos DB will be presented.

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PL06
MONITORING ACQUISITION OF GLYCOPROTEIN TERTIARY AND QUATERNARY STRUCTURES IN THE ENDOPLASMIC RETICULUM.

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The folding status of glycoproteins is stringently controlled in the endoplasmic reticulum (ER) by a glucosyltranferase (GT) that creates monoglucosylated structures recognized by ER resident lectins (calnexin and calreticulin). GT serves as a folding sensor as it only glucosylates glycoproteins not displaying their native structures. To explore the structural determinants for GT-mediated glucosylation we used as acceptor substrates a family of chemically glycosylated proteins derived from chymotrypsin inhibitor 2. Structural characterization of species showing higher glucose acceptor capacity indicated that GT recognizes solvent accessible hydrophobic patches in molten globule-like conformers mimicking glycoprotein advanced folding stages. Further, to determine if the GT-derived quality control is operative with conformers displaying structures closely resembling those of native species and that, therefore, no alternative ER retaining mechanisms are required when glycoproteins approach their native folding, we generated and used as acceptor substrates neoglycoproteins bearing conformations mimicking the last stage folding structures adopted by glycoproteins entering the ER lumen. Results obtained demonstrate that GT is an exquisite sensor of glycoprotein conformations able to recognize folding intermediates only slightly differing from native species. Finally, results obtained using the isolated subunits of a homotetrameric glycoprotein (soybean agglutinin) as acceptor substrate showed that GT is also able to recognize hydrophobic interfaces exposed by properly folded subunits not yet forming part of multimeric complexes, thus ensuring ER retention of incompletely assembled multimeric species. The same mechanism operates, therefore, to monitor glycoprotein acquisition of tertiary and quaternary structures.

PL07
GLYYCOSPHINGOLIPIDS OF CELLULAR MEMBRANES –METABOLISM AND FUNCTIONS

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Glycosphingolipids (GSL) are membrane components of eukaryotic cell surfaces. Their biosynthesis is controlled by enzymes bound to intracellular membranes which generate a cell-type-specific molecular diversity. Glycosyltransferases interact with membrane-bound GSL substrates within the plane of the lipid bilayer. Only few glycosyltransferases of limited specificity are needed to generate complex ganglioside patterns. This combinatorial scheme of ganglioside biosynthesis is supported by the analysis of knock out mice.
The constitutive degradation of GSL takes place on the surface of intra-endosomal and intra-lysosomal membrane structures. During endocytosis, these intra-lysosomal membranes are formed and prepared for digestion by a lipid sorting process, during which their cholesterol content decreases and the concentration of the negatively charged bis(monoacylglycero)phosphate (BMP) increases. Lysosomal GSL degradation requires the presence of water-soluble acid exohydrolases, sphingolipid activator proteins (saposins and GM2 activator protein), and anionic phospholipids like BMP. Sphingolipid activator proteins are membrane-perturbing and lipid-binding proteins which present the lipids to the degrading enzymes, thereby overcoming the water-lipid barrier. Their inherited deficiency leads to sphingolipid- and membrane-storage diseases. Sphingolipid activator proteins not only facilitate glycolipid digestion, but also act as glycolipid transfer proteins facilitating the association of glycolipid antigens with immunoreceptors of the CD1 family. Recently, a new concept for the generation of the extracellular water-permeability barrier in the stratum corneum of land-dwelling animals was initiated by the discovery of protein-bound ω-hydroxylated glucosylceramides in the skin.

PL08
EMERGING VIEWS AND CONCEPTS TO ADDRESS THE CHALLENGES INHERENT TO THE STUDY OF GLYCAN STRUCTURE-FUNCTION RELATIONSHIPS

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The ‘omics approach to studying glycans – *glycomics,* provides an integrated *systems* approach to the structure-function relationships of these complex molecules. The need for an integrated approach to study glycans stems from the fact that glycans, being present at the cell-extracellular matrix interface, regulate fundamental biological processes. In comparison with genomics and proteomics, the advancement of glycomics has faced unique challenges in pursuit of developing analytical and biochemical tools as well as biological read outs to investigate glycan structure-function relationships. Glycans are more diverse in terms of chemical structure and information density than are DNA and proteins. This diversity arises from glycans’ complex non-template based biosynthesis which involves several enzymes in addition to their isoforms. Consequently, glycans are expressed as an ‘ensemble’ of structures that mediate function. Moreover, unlike protein-protein interactions, which can be generally viewed as ‘digital’ in regulating function, glycan – protein interactions impinge on biological functions in a more ‘analog’ fashion that can in turn “fine tune” a biological response. This fine tuning by glycans is achieved through the graded affinity, avidity and multivalency of their interactions. By highlighting both branched glycans and linear glycosaminoglycans as examples this talk will expound on the emerging views and approaches aimed at addressing some of these challenges.

PL09
SYNTHETIC TOOLS TO ELUCIDATE THE BIOLOGY OF GLYCOCONJUGATES

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The importance of cell surface oligosaccharides and glycosaminoglycans in signal transduction processes of biomedical significance is now well established. A major impediment to the rapidly growing field of molecular glycobiology was the lack of pure, structurally defined carbohydrates and glycoconjugates. Described is a comprehensive program involving all aspects of automated oligosaccharide assembly on solid support. [1]
Based on the synthetic platform, a suite of tools for glycobiologists has been developed that includes carbohydrate microarrays, fluorescently labeled oligosaccharides for imaging studies as well as affinity columns and other synthetic tools. [2]
Using the specific binding of certain bacteria to particular sugars was used to develop a visual detection system to test body fluids and water for the presence of pathogens including *E. coli*. [3] To improve the detection of several pathogens at the same time, carbohydrate arrays were utilized to measure binding of cells to sugars. [4]
Described will be the development of carbohydrate based vaccines against a series of diseases including HIV, leishmaniasis and tuberculosis. Particular emphasis will be placed on the example of an anti-toxin malaria vaccine that is currently in preclinical development. [5]
Finally, the use of fully synthetic glycolipids from P. falciparum*, [6]* T. gondii *and* M. tuberculosis *to elucidate lipid signalling, receptors involved in glycolipid recognition and to understand natural immunity to these diseases will be discussed. The biosynthesis of glycolipids is being studied en*

route to the development of selective inhibitors as drugs against pathogenic organisms.

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PL10
DYNAMIC AND STRUCTURAL PROPERTIES OF GLYCOSPHINGOLIPIDS AS DRIVING FORCES TO THE FORMATION OF MEMBRANE DOMAINS.

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Cell membrane components are not randomly distributed but are organised as specialized domains, that have been shown to display dynamic properties and to be involved in membrane-associated events such as cell signaling, cell adhesion and protein sorting. This explains the huge interest of scientists to membrane lipid domains, as proven by the continuously growing number of papers related to this topic.
A common feature of cell membrane domains is their peculiar lipid composition, being they enriched in sphingolipids and cholesterol, and their low content of proteins. Theoretical considerations and experimental data suggest that a few properties own of glycosphingolipids, such as their geometrical properties, their capability to form a network of hydrogen bonds at the water-lipid interface of cell plasma membrane, their capability to interact with water and their specific content of saturated alkyl chains, play an important role in the formation of cell membrane lipid domains. These features, that differentiate glycosphingolipids from the other lipids in the membranes, allow self-interaction, phase separation and promote co-segregation with cholesterol, thus forming less fluid membrane zones capable to segregate proteins and modulate their cellular functions.

PL11
MULTIPLE DIFFERENCES IN SIALIC ACID BIOLOGY BETWEEN HUMANS AND GREAT APES

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Human proteins are >99% identical to those of chimpanzees and bonobos, our closest evolutionary relatives. We have discovered multiple genetic and biochemical differences between humans and great apes in relationship to Sialic acids (Sias). One major Sia called Neu5Gc is widely expressed in mammals including apes, but not humans. This results from an inactivating mutation in the human *CMAH* gene, mediated by an *Alu* replacement about 3 million years ago. Additional human-specific changes in Sia biology include: a build-up of the precursor Sia Neu5Ac; differences in expression and distribution of Siglec-1 (sialoadhesin), a macrophage receptor recognizing Neu5Ac and not Neu5Gc; a mutation in *SIGLECXII*, changing an arginine required for Neu5Gc recognition in apes; Deletion of human *SIGLEC13*; human-specific gene conversion of *SIGLEC11* changing expression and function; enhanced alpha2-6Sia expression on some cell types; and, changes in Neu5Gc versus Neu5Ac preferences and expression patterns of some Siglecs. As <60 genes are involved in synthesis, transfer, recognition and turnover of Sias, these changes are likely not due to chance. Implications for humans include susceptibility or resistance to certain microbial pathogens; effects on immune system reactivity; potential effects on placental function; expression of Neu5Gc as a "foreign" antigen in cancers; consequences of human dietary intake of Neu5Gc from animal foods such as red meat; the human immune reaction against Neu5Gc, and issues in biotechnology and xenotransplantation. This lecture will summarize these findings, with attention to the Chimpanzee Genome Project as a resource to explore Sia biology differences between humans and other primates.

LECTURES

L001
STRUCTURAL DIVERSITY AND CARBOHYDRATE BINDING PRINCIPLES IN LECTINS

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Crystal structures of members of the different animal and plant lectin families have revealed a wide variety of lectin folds and carbohydrate binding site architectures. Despite this large variability, a number of interesting cases of convergent as well as divergent evolution among plant, animal and bacterial lectins are noted. These similarities exist at the level of the protein fold, the architecture of the binding site as well as quaternary association and may be derived from similar functional needs. The similarities and differences in the architecture of lectin carbohydrate binding sites and quaternary structures will be discussed, with emphasis on the legume lectin family. Using the *Pterocapus angolensis* lectin as an example, we will examine how structure relates to thermodynamics of binding and how structure, stability and function are interlinked.

L002
GLYCOSYLTRANSFERASE MECHANISM: METAL IONS, BASIC RESIDUES AND CATALYSIS

JE Pak, P Arnoux, D Ma, P Sivarajah, S Zhou, X Xing and JM Rini.
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The Core 2 β1,6-*N*-acetylglucosaminyltransferase (C2GnT) transfers GlcNAc from UDP-GlcNAc to the core 1 acceptor (Galβ1,3GalNAcβ-O) to generate core 2 branched oligosaccharides (Galβ1,3[GlcNAcβ1,6]GalNAcβ-O). Here we report the x-ray crystal structure of C2GnT and its Galβ1,3GalNAc complex at 2.0 and 2.7 Å resolution, respectively. The structures show that the enzyme possesses the GT-A fold, the first example of a nucleoside diphosphate sugar utilizing glycosyltransferase of this type. The enzyme is metal ion independent and does not contain a DXD motif or bound metal ion. Structural alignment suggests that the catalytic role played by metal ion in other GT-A enzymes is replaced by the side chains of basic amino acids in C2GnT. The use of basic side chains in this way is a feature of GT-B type glycosyltransferases, enzymes that are otherwise fundamentally different in structure. The structures have also provided a detailed description of the determinants of acceptor specificity, identification of the catalytic base, as well as an explanation for the oxidative inactivation shown by C2GnT.

L003
INTERACTIONS OF CALRETICULIN, A MOLECULAR CHAPERONE IN THE ENDOPLASMIC RETICULUM, WITH ITS SUBSTRATE

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Calreticulin is a molecular chaperone found in the endoplasmic reticulum in eukaryotes, whose interaction with N-glycosylated polypeptides is mediated by the glycan, $Glc_1Man_{7\text{-}9}GlcNAc_2$, present on the target glycoproteins. We have determined the real time association kinetics for the interaction of monoglucosyl IgG substrate with calreticulin by surface plasmon resonance. The data obtained are consistent with a single-step bimolecular reaction and the derived activation parameters demonstrate that the reaction is enthalpically driven also attested to by the direct determination of thermodynamic parameters using isothermal titration calorimetry. The studies demonstrate that calreticulin has only one site per molecule for binding its complementary glucosylated ligands. While the binding of glucose by itself is barely detectable, it increases strikingly when glucose occurs in α-1,3 linkage to ManαMe as in Glcα1-3ManαMe. The binding constant increases by 25 fold in going from di- to trisaccharide and doubles in going from tri- to tetrasaccharide, demonstrating that the entire Glcα1-3Manα1-2Manα1-2ManαMe structure of the oligosaccharide is recognized by calreticulin. Thermodynamic parameters thus obtained are supported by modeling studies, which show that increased number of hydrogen bonds and van der Waals interactions occur as the size of the oligosaccharide is increased. The role of the residues implicated in the binding of the oligosaccharides was probed further by site specifically mutagenizing them. These studies show that the residues that form the primary (glucose) binding site show far less tolerance to mutations than the residues that interact with the mannosyl residues of the glycan. Based on these and other studies we propose that calreticulin assists in the folding of glycoproteins by kinetically partitioning them.

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L004
CHONDROITIN 4-SULFATE-MEDIATED ADHERENCE OF *PLASMODIUM FALCIPARUM* IN PREGNANCY-ASSOCIATED MALARIA.

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A key feature of *Plasmodium falciparum* infection is the adherence of the parasite-infected red blood cells (IRBCs) in the microvascular capillaries of vital organs. This binding is mediated to by the endothelial cell surface adhesion molecules and the adhesive proteins expressed on the IRBC surface. By adulthood, people in endemic areas, regardless of gender, produce inhibitory antibodies to the adhesive proteins and thus avoid IRBC adherence. However, in the case of women, during pregnancy, the IRBCs with adherent specificity to chondroitin 4-sulfate (C4S) selectively adhere in the placenta and multiply. Thus extensive accumulation of IRBCs in the placenta leads to poor pregnancy outcomes. We have demonstrated that a uniquely low-sulfated, chondroitin sulfate proteoglycan (CSPG) localized in the intervillous space of the placenta is the receptor for IRBC adherence. Optimal IRBC binding requires a minimum dodecasaccharide motif and participation of 4-sulfated and 4-nonsulfated disaccharides of C4S. The sulfate and hydroxyl groups at C-4 of GalNAc and the carboxyl group of uronic acid are critical for the IRBC adhesion, whereas the *N*-acetyl group of GalNAc is not involved. In the C4S chains of the placental CSPG, the majority of the sulfated disaccharides are clustered and these are the regions that mediate the IRBC adherence. In an effort to identify the parasite adhesive protein(s), we have prepared photo-affinity cross-linking probes by replacing *N*-acetyl groups of C4S dodecasaccharides with iodinatable azido group-containing benzoyl moieties. Preliminary results indicate that a novel low molecular weight protein is involved in IRBC binding to C4S. Supported by AI45086 from NIAID, NIH.

L005
STRUCTURAL BIOLOGY OF JACALIN-LIKE LECTINS

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The first lectin to be shown to have the β-prism I fold was the galactose-specific jacalin, one of the two lectins from jack fruit seeds. The other lectin, artocarpin, also assumes the same fold, although it is mannose-specific. Both the lectins are tetrameric. The binding sites of the two lectins have been thoroughly characterized. This characterization led to the elucidation of post-translational modification, variation of loop length and interactions involving aromatic groups as strategies for generating or modifying carbohydrate specificity. Since then, the structures of several other plant lectins with β-prism I fold, all from dicots, have been reported. They exihibit a variety of quaternary structures and variations in oligosaccharide specificity based on a single primary site located on one of the three Greek keys that constitute the β-prism I fold. Most recently the structure of banana lectin, another lectin with this fold, has been determined. In it, two Greek keys carry one primary binding site each, with a common secondary site in the third key. This can be explained in terms of the evolution of jacalin-like lectins through successive gene duplication and fusion of an ansecstral sugar-binding Greek key motif. Banana is a manocot and, unlike in the jacalin-like lectins form dicots, the three motifs have not diverged enough to obliterate sequence similarity among them. The geometrical disposition of the two primary binding sites and the common secondary binding site is such that it provides a structural rationale for the known specificity of banana lectin for branched α- mannans.

L006
CELL SPECIFIC DELETION OF GLUCOSYLCERAMIDE SYNTHASE IN BRAIN LEADS TO SEVERE NEURAL DEFECTS AFTER BIRTH

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Glycosphingolipids are believed to play important roles in a variety of cellular processes including cell-cell interactions, cell-adhesion, differentiation, intra-cellular transport and signaling.

Sialic acid containing glycosphingolipids, *i.e.* gangliosides, constitute a major component of neuronal cells and are thought to be essential for brain function. UDP-glucose:ceramide glucosyltransferase (Ugcg) catalyzes the initial step of glycosphingolipid (GSL) biosynthesis. The enzyme was initially cloned by the group of Hirabayashi (1) and a total knock-out of the Ugcg-gene in mice led to embryonic lethality during gastrulation at stage E6.5 to E7.5 (2).

In order to gain insight into the role of GSLs in brain development and function, a cell specific disruption of Ugcg was performed using the Cre/loxP-system. LoxP-flanked Ugcg-mice were generated and crossed with nestin-cre mice. The nestin-promoted gene deletion in neural cells was indicated by the absence of virtually all glucosylceramide based GSLs already at stage E15.5.

Shortly after birth mice showed dysfunction of cerebellum and peripheral nerves, associated with structural defects. Axon-branching of Purkinje cells was significantly reduced. In primary cultures of neurons dendritic complexity was clearly diminished, while pruning occurred. Myelin sheaths of peripheral nerves were broadened and focally severely disorganized. GSL deficiency also led to a downregulation of gene expression sets involved in brain development and homeostasis. Mice died approximately 3 weeks after birth.

The pronounced neurologic symptoms in mice with neuronal specific deficiency of glucosylceramide synthesis demonstrate

that GlcCer-derived GSL may not serve functions essential for early brain development. They are, however, required for neuron differentiation and brain maturation.

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L007
BIOLOGICAL FUNCTIONS OF SULFOGLYCOLIPIDS REVEALED BY GENE DISRUPTION OF CEREBROSIDE SULFOTRANSFERASE

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Mammalian sulfoglycolipids are comprised of two major members, sulfatide (SO_3-3Gal-ceramide) and seminolipid (SO_3-3Gal-alkylacylglycerol). Sulfatide is abundant in the myelin sheath and seminolipid is expressed on the spermatogenic cells. The carbohydrate moiety of sulfatide and seminolipid has the same structure and is biosynthesized via sequential reactions catalyzed by common enzymes: ceramide galactosyltransferase (CGT, EC 2.4.1.45) and cerebroside sulfotransferase (CST, EC 2.8.2.11). CST-deficient mice generated by gene targeting completely lack sulfatide and seminolipid all over the body. CST-null mice manifest some neurological disorders due to myelin dysfunction, an aberrant enhancement of oligodendrocyte terminal differentiation, and an arrest of spermatogenesis, indicating that sulfation of glycolipids is essential for myelin formation and spermatogenesis. However, the molecular mechanisms in which sulfoglycolipids are involved during these biological processes remain unknown. To address the issue of which side of the cell function, germ cells or Sertoli cells, is deteriorated in this mutant mouse, we employed a germ-cell transplantation analysis, in which wild-type spermatogonia were injected into the seminiferous tubules of CST-null testis. The transplanted green fluorescent protein-positive cells generated colonies and spermatogenesis proceeded over meiosis in the mutant testis, suggesting that the defect is in the germ cell side. The fact that seminolipid is recovered in detergent-insoluble floating membrane fractions suggests that seminolipid is included in the lipid rafts of germ cells. Since these microdomains are proposed to serve as platforms within the plasma membrane for receptor signaling and trafficking, seminolipid may contribute to the organization of such functional platforms on the germ cells.

L008
N-ACETYLGLUCOSAMINYLTRANSFERASE I-NULL DROSOPHILA MELANOGASTER IS UNABLE TO COMPETE FOR SURVIVAL IN THE PRESENCE OF WILD TYPE FLIES

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Introduction. UDP-GlcNAc:Alpha-3-D-mannoside beta-1,2-*N*-acetylglucosaminyltransferase I (GnT I) controls the synthesis of hybrid, complex and paucimannose *N*-glycans. *Drosophila melanogaster* makes paucimannose but little or no hybrid nor complex *N*-glycans. The single GnT I gene in flies has been cloned and expressed (Sarkar M, Schachter H. Biol Chem 2001, 382:209-217).
Methods. GnT I-null *D.melanogaster* lines were obtained by imprecise excision of a P-element located 546 bp upstream of the start codon; a 1301 bp deletion was produced downstream of the P-element. No flanking genes were disrupted.
Results. GnT I -/- adults were recovered only when animals were removed from the vial at the larval stage and allowed to develop together with a limited number of mutant larvae. Mutant embryos eclosed normally but had a significantly reduced life span (98% dead within 15 days; 80 days for heterozygotes). GnT I -/- adults are viable with a normal external morphology. Locomotor activity (open grid method) showed that -/- flies are significantly more sluggish than wild type flies. No eggs were obtained on attempts to mate mutant males and females. Extracts of GnT I -/- flies showed no GnT I activity. Mass spectrometric analysis of these extracts showed dramatic changes in *N*-glycans compatible with GnT I lack.
Conclusions. The data indicate that GnT I-dependent *N*-glycans are required for normal development of the nervous system of the fly.
Support by the Canadian Institutes of Health Research (CIHR) and the Canadian Protein Engineering Network Centre of Excellence (PENCE).

L009
SYSTEMATIC GENE KNOCK-OUT OF HUMAN GLYCOGENE ORTHOLOGS IN THE NEMATODE *C. ELEGANS* USING RNAI AND DELETION MUTAGENESIS

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The nematode *Caenorhabditis elegans* is an ideal multicellular model organism for studying the roles of glycome-related genes (glycogenes) in development and morphogenesis. All the cell lineages of 959 somatic cells and all the neuronal connectivity of 302 neurons are completely known. Development of any single cell of embryos can be monitored and recorded with four-dimensional microscopy, and any defects caused by glycogene mutation can be detected easily. Transgenic animals can be made in a few days, and deletion mutant animal of any gene can be isolated also in a few days. The mutant worms can be frozen stocked. RNAi technology discovered in this animal is also useful in knocking out of most of the genes. With these advantages in mind, we have been trying to knock out as many glycogenes as possible in our functional glycomics project.
In this study, with the aid of sophisticated bioinformatic technology, we searched the genome of *C. elegans* for human glycogene orthologs and found 145 different potential orthologous genes. RNAi experiments of all these genes were performed, and RNAi of 20% or more of these glycogene orthologs resulted in severe phenotypes including embryonic lethality and abnormal morphogenesis. Isolation of deletion mutants of these genes are also in progress, and deletion mutants often results in more severe phenotypes than in RNAi experiments.
All these results indicate the importance of glycogenes in development and morphogenesis, and detailed study on these genes will throw light on the new roles of glycoconjugates in multicellular organisms.

L010
ALTERATION OF CORNEAL EXTRACELLULAR MATRIX STRUCTURE IN *CHST5*-NULL MICE, WHICH LACK A CARBOHYDRATE SULFOTRANSFERASE FOR KERATAN SULFATE PRODUCTION

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Keratan sulfate (KS) proteoglycans are largely found in cornea and cartilage, and are suggested to have biological function for the maintenance of corneal transparency. Previously we identified *CHST6* as a gene encoding a carbohydrate sulfotransferase for keratan sulfate production, and found that mutations on *CHST6* result in macular corneal dystrophy (MCD), a hereditary disease in which the patients develop clouding of the cornea due to abnormal carbohydrate deposits. In mouse, *Chst5* is the authologue of human *CHST6* and encodes a sulfotransferase (mIGn6ST, also called GlcNAcT-3 and GST4) that also has activity for KS sulfation. Here we generated a mouse strain that lacks mIGn6ST activity and observed morphological and biological phenotypes. Highly sulfated KS, which is detected by 5D4 antibody, is not detected in the cornea of *Chst5*-null mice, indicating mIGn6ST is one of the responsible enzymes for corneal KS production. Unlike the typical phenotype found in MCD patients, *Chst5*-null mice didn't show obvious opacity in their corneas even at 1-year old. By X-ray fibre diffraction analysis, we found altered collagen fibril organization in the corneal extracellular matrix of *Chst5*-null mice. Collagen fibrils in *Chst5*-null corneas are more widely spaced and more disorganized than those wild type corneas. Some of these features are also reported in MCD corneas suggesting that in some respects *Chst5*-null mouse is an animal model of MCD in humans. These results found in *Chst5*-null mice demonstrate the important biological function of KS carbohydrate in the maintenance of corneal extracellular matrix structure.

L011
DEVELOPMENTAL EXPRESSION OF CARBOHYDRATE EPITOPES CONTRIBUTES TO MOLECULAR MIMICRY DURING THE LIFE-CYCLE OF *SCHISTOSOMA MANSONI*

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Introduction. Schistosomiasis is one of the most important parasitic diseases with 200 million people infected worldwide. The causative agents of this disease are trematodes of the genus *Schistosoma* which have a complex life cycle including human beings as definitive host and freshwater snails as intermediate host. Since schistosomes have to invade and to survive in different hosts, the question arises as to whether these worms are utilizing molecular mimicry as a survival strategy. Therefore, we have analyzed *S. mansoni* glycosphingolipids expressed in different life cycle stages as

well as glycoconjugates of its intermediate host *Biomphalaria glabrata*.
Experimental approach. Glycans were released by ceramide glycanase or PNGase F and analyzed by mass spectrometry. In parallel, neoglycolipids were produced and characterized by ELISA.
Results. The results revealed a stage-specific expression of glycosphingolipids decorated with definitive host-like Lewis X as well as parasite-specific pseudo-Lewis Y determinants in the cercarial stage. Intriguingly, both carbohydrate motifs are recognized by the human C-type lectin DC-SIGN. In the egg stage glycolipids exhibit different terminal carbohydrate epitopes including a Fuc(α1-3)GalNAc-motif. Structural analyses of haemolymph glycoprotein-N-glycans from *B. glabrata* revealed the presence of several carbohydrate epitopes shared by glycoconjugates from both *S. mansoni* eggs and its intermediate host including the Fuc(α1-3)GalNAc-epitope.
Conclusions. Our results demonstrate that schistosomes imitate host-like carbohydrate epitopes in the cercarial as well as in the egg stage which may be considered as a first indication of molecular mimicry used by the parasite for successful invasion and survival in its hosts.

L012
THE SUPPRESSION OF GALACTOSE METABOLISM IN *TRYPANOSOMA CRUZI* EPIMASTIGOTES CAUSES CHANGES IN CELL SURFACE ARCHITECTURE AND CELL MORPHOLOGY.

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The cell surface of the epimastigote form of *Trypanosoma cruzi* is covered by glycoconjugates rich in galactose. Galactose transport is not possible through its hexose transporter, so epimerization of UDP-glucose to UDP-galactose by UDP-glucose 4'-epimerase may be the only way that the parasite can obtain this sugar. The epimerase is encoded by the *TcGALE* gene and epimastigote form *T. cruzi TcGALE* single-allele knockouts were constructed. The inability to create a double-allele knockout alludes to the importance of the expression of this gene to the cell. The single-allele knockouts exhibited gross morphological changes as viewed by electron microscopy, with phenotypes including agglutination, shortened flagella, increased incidence of spheromastigotes, and a novel walnut-like appearance. Furthermore, fluorescence microscopy indicated an increase in sialic acid at the cell surface, which was surprising given the significant reduction in the surface glycoconjugates of galactose (the sugar through which sialic acid is joined to these molecules). The major loss of galactose in mucins as opposed to glycoinositolphospholipids may indicate the importance of this glycocalyx as a minimum for parasite survival. This apparent haploid insufficiency suggests that epimerase levels are close to limiting in the cell and may be exploited as a potential drug target.

L013
HEPARIN-PROTEIN INTERACTIONS

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Heparin, an anticoagulant, acts on the coagulation cascade through binding and activation of the ATIII.[1] Heparin and the related heparan sulfate are glycosaminoglycans (GAGs) with repeating glucosamine and uronic acid residues. They exert their biological activities through the localization, stabilization, activation or inactivation of interacting proteins. These interactions play important roles in normal physiology and are also involved in pathological processes. Although these interactions are of great biological importance, structural requirements for protein-GAG binding have not been well characterized.[2] Both the presence of specifically positioned sulfo groups on the GAG and consensus sequences clustering of basic residues in the protein have been proposed to be important in GAG-protein interactions. The interaction of heparin with various proteins including, growth factors,[3] virus envelope proteins,[4] chemokines,[5] serine protease inhibitors and others are presented. The structure of the various protein-binding sites for their GAG ligand, the specificity and strength of binding are of physiological and pharmacological relevance.

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L014
REGULATION OF TUMOR ANGIOGENESIS BY PERLECAN

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Emerging evidence indicates that basement membrane proteoglycans play both pro- and anti-angiogenic roles. The former is mediated primarily by the enhancement of FGF activity by heparan sulfate side chains and specific microsenquences within them. The latter is mediated by processed forms of their protein cores, especially C-terminal modules that interact with endothelial cell receptors. One such factor is endorepellin which encompasses the C-terminal domain V of human perlecan. Endorepellin is active at nanomolar concentrations and affects macrovascular and microvascular endothelial cells by directly binding to the I domain of the α2β1 integrin. The endorepellin/α2β1 interaction triggers a unique signaling pathway that evokes a rapid increase in the second messenger cAMP, sustained activation of the two proximal kinases, PKA and FAK, transient activation of p38 MAPK and Hsp27, followed by a rapid down-regulation of the latter proteins. This biological axis leads to disassembly of actin stress fibers and focal adhesions, and ultimately causes a block of endothelial cell migration and angiogenesis. Using a tumor xenograft model of squamous cell carcinoma, we show that following systemic injection, endorepellin homes to the tumor neovasculature and significantly suppresses tumor growth and metabolic activity as measured by positron emission tomography. As perlecan is present in both endothelial and smooth muscle cell basement membranes, anti-angiogenic endorepellin could be liberated by the concerted action of proteases at sites of active vascular remodeling and tumor invasion.

L015
HEPARAN SULFATE AND HEPARANASE: PARTNERS IN DRIVING GROWTH AND METASTASIS OF OSTEOLYTIC TUMORS

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Heparan sulfate proteoglycans act as powerful regulators of cell behavior by mediating cell adhesion and by acting as co-receptors for growth factors that regulate cell signaling. We have discovered that the syndecan-1 heparan sulfate proteoglycan acts in vivo to promote the growth and metastasis of myeloma and breast tumors; two tumors that home to, and grow within, bone. Using in vivo models of these cancers, we find that the growth-promoting effect of syndecan-1 resides in the shed form of the molecule which acts to favorably condition the tumor microenvironment. Moreover, the interplay between heparan sulfate and the extracellular enzyme heparanase also has important regulatory implications. Heparanase, which releases heparan sulfate-bound growth factors and generates highly active heparan sulfate fragments, promotes myeloma growth, angiogenesis and spontaneous metastasis to bone. In an in vivo model of breast cancer we find that heparanase expression by a primary tumor in the mammary fat pad leads to bone resorption in the absence of detectable tumor within the bone. This data reveals that heparanase can have both local and distal effects that contribute to the morbidity of cancer. Together our findings demonstrate that heparan sulfate and heparanase within the tumor microenvironment are critical regulators of malignant behavior and thus represent important therapeutic targets for treatment of osteolytic tumors.

L016
HEPARANASE AS A TARGET FOR THE DEVELOPMENT OF ANTI-CANCER DRUGS

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Heparanase is an endo-β-D-glucuronidase involved in cleavage of heparan sulfate (HS) and thereby in degradation and remodeling of the extracellular matrix (ECM). Heparanase activity has been correlated with the metastatic potential of tumor-derived cell types and with cell invasion associated with autoimmunity, inflammation, and angiogenesis. Heparanase upregulation often correlates with increased tumor vascularity and poor postoperative survival of cancer patients. These observations and the unexpected identification of a single functional heparanase, suggest that the enzyme is a promising target for anti-cancer drug development. We investigated the heparanase inhibiting effect of heparin derivatives differing in degrees of 2-O- and 6-O-sulfation, N-acetylation, and glycol-splitting of nonsulfated uronic acid residues. N-desulfation/N-acetylation involved a marked decrease in the inhibitory activity for degrees of N-acetylation higher than 50%, suggesting that at least one NSO_3 group per a disaccharide unit is involved in interaction with heparanase. On the other hand, glycol-splitting of preexisting or of both preexisting and chemically generated nonsulfated uronic acids dramatically increased the heparanase-inhibiting activity, irrespective of the degree of N-acetylation. Indeed, N-acetylated heparins in their glycol-split forms inhibited heparanase as effectively as the corresponding N-sulfated derivatives. Glycol-split N-acetyl heparins were resistant to cleavage by heparanase, did not release FGF-2 from ECM and failed to stimulate its mitogenic activity. The combination of high inhibition of heparanase and low release/potentiation of ECM-bound growth factors points to N-acetylated, glycol-split heparins as potential anti-angiogenic and anti-metastatic agents, more effective and specific than their counterparts

with unmodified backbones. Heparanase gene silencing, brought about by anti-heparanase ribozyme or siRNA resulted in a marked inhibition of tumor metastasis and angiogenesis in mouse melanoma and lymphoma models. A potent inhibition of heparanase activity was also exerted by a peptide corresponding to the heparin-binding domain of heparanase and by antibodies directed against this region. Identification of amino acids and regions essential for proteolytic processing and activation of pro-heparanase, as well as for substrate recognition and interaction between the 8 and 50 kDa subunits of the enzyme, yielded a 3D model of the heparanase molecule, currently used to design additional inhibitory strategies.

L017
STRUCTURAL INSIGHTS INTO THE HEPARIN ACTIVATION MECHANISMS OF SERPINS

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The family of serine protease inhibitors known as the serpins has evolved an extraordinary mechanism to inhibit proteases which renders them uniquely qualified to control the proteolytic pathways essential to life. The mechanism is best described as a spring-loaded mousetrap, where nibbling of the peptide loop bait springs the trap and crushes the unsuspecting protease. The complexity of the serpin mechanism provides many advantages over the simpler lock-and-key type mechanism utilized by other serine protease inhibitor families: serpins provide stoichiometric, irreversible inhibition, and the dependence on serpin and protease conformational change is exploited for regulation, signaling and clearance. Regulation of serpin activity is best illustrated by the heparin activation mechanisms. Twenty-five serpins have been identified in the human genome, and five are known to bind heparin-like glycosaminoglycans: antithrombin, heparin cofactor II, plasminogen activator inhibitor-1, protease nexin 1, and protein C inhibitor. All are implicated in hemostatic or fibrinolytic mechanisms, but also have important extravascular functions. AT is the principal inhibitor of the coagulation proteases, and is the effector molecule for therapeutic heparin. Its heparin binding mechanism is the best characterized of the five, and therefore AT serves as the prototype heparin binding serpin. Recent crystallographic structures have shed new light on how heparin binding fine-tunes the inhibitory mechanism of serpins, and how seemingly disparate mechanisms of heparin binding and activation can share critical elements. This lecture describes recent developments in our understanding of the molecular mechanisms involved in the heparin activation of serpins.

L018
CONFORMATIONAL TRANSITIONS INDUCED IN HEPARIN OLIGOSACCHARIDES BY BINDING WITH ANTITHROMBIN IN SOLUTION.

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Heparin is a sulfated glycosaminoglycan prevalently constituted by repeating disaccharide units made up of α-1,4-linked 2-O-sulfated L-iduronic acid ($IdoA2SO_3$) and N,6-O-sulfated D-glucosamine ($GlcNSO_36SO_3$). "Irregular" sequences containing N-acetyl D-glucosamine (GlcNAc) and β-D-glucuronic acid (GlcA), as well as other undersulfated residues, contribute to various biological activities of heparin. The pentasaccharide sequence AGA*IA ($GlcNAc/SO_36SO_3$-GlcA-$GlcNSO_33,6SO_3$-$IdoA2SO_3$-$GlcNSO_3,6SO_3$), which constitutes the active site for antithrombin (AT), is essential for the expression of high anticoagulant and antithrombotic activities.
The present study deals with the conformation in solution of heparin oligosaccharides containing the AGA*IA sequence in different positions along the chain and was focused on IdoA both within and outside the AGA*IA sequence. Analysis of NMR parameters (NOEs and transferred NOEs, proton and carbon chemical shifts and coupling constants) for two octasaccharides clearly indicated that, as previously observed for the pentasaccharide,[1] out of the two ($^1C_4$ and $^2S_0$) conformations present in dynamic equilibrium in the free state for the $IdoA2SO_3$ residue, the protein selects the $^2S_0$ form. Notably, the $^2S_0$ conformation is taken up also for the nonsulfated IdoA preceeding AGA*IA, which in the absence of AT is largely in the $^1C_4$ form. These results suggest a possible biological role for that residue in modulating anticoagulant and antithrombotic properties of heparin chains. They also further support the concept that heparin-binding proteins may influence the conformational equilibrium of iduronate residues of sequences directly or indirectly involved in binding, and select one of their equienergetic conformations for best fitting in the complex.[2] As recently shown also for a heparin tetrasaccharide complexed with FGF-2,[3] the selected form usually corresponds to the one prevalent in the free state. However, complete reversal of the conformation preferred in the free state was demonstrated here for the first time.

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L019
CONFORMATIONAL FLEXIBILITY OF A SYNTHETIC HEXASACCHARIDE BOUND TO FIBROBLAST GROWTH FACTOR-1

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Fibroblast growth factors (FGF) are proteins involved in the extracellular regulation of biological processes related with cell proliferation. They activate the signalling through the formation of a ternary complex together with heparan sulphate and FGFR (their specific membrane receptor). We have been investigating the molecular bases of the heparin-FGF interaction using synthetic oligosaccharides with different sulphation patterns by comparing their efficiency in the induction of FGF-1 mediated mitogenesis. The most active compound was a hexasaccharide with a substitution pattern designed to yield a three dimensional arrangement of the sulphate groups non adequate for FGF *trans*-aggregation, the resulting sequence has not been detected in natural glycosaminoglycans. This oligosaccharide retains all the essential features of the 3-dimensional structure of the natural heparin: the 3-D structure of the backbone and the conformational equilibrium of the iduronate residues. By means of ^{13}C double filtered NMR experiments, achieved using stable complex of double-labelled [^{15}N,^{13}C] FGF-1-oligosaccharide and the hexasaccharide, it has been possible to detect the ^{1}H of the bound carbohydrate without interference from the protein signals. The chemical shifts of the bound ligand were very similar to those observed for the free. The NOE cross-peaks patterns and intensities were also comparable to the free compound indicating a similar 3D structure. Moreover, the H2-H5 cross-peaks, indicative of the $^{2}S_{O}$ conformation of iduronate residues, retained the intensities pattern from the free conformation. These results are compatible with the formation of a flexible complex in which FGF-1 does not induce conformational selection of the iduronate rings.

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L020
MODELLING OF HEPARANSE PEPTIDES WITH HEPARAN SULFATE OLIGOSACCHARIDES.

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Heparanase is an endo-β-D-glucoronidase that degrades heparan sulfate (HS) proteoglycans on cell surfaces and in the extracellular matrix. Modelling of interactions between the heparanase binding domain Lys_{158}-Asp_{171} and different HS pentasaccharides have been performed in the present study. Both molecular mechanics and Monte Carlo conformational search were used to obtain 3D structure of the complexes between seven pentasaccharides and the peptide. The analysis of these complexes enabled description of the interaction among sulphate and carboxylate groups in oligosaccharides and positively charged groups in the peptide. Furthermore, DFT calculations of selected model compounds were aimed at detailed understanding of the role of Na counter-ions in interactions with negatively charged groups in saccharides. The analysis of pentasaccharide GlcN,6S-GlcA-GlcN,3,6S-IdoA2S-GlcN,6S-OMe – peptide interactions showed that, in the lowest energy complex, N-sulfate groups in both reducing and the non-reducing end glucosamine residues, the 6-O-sulfate in the GlcN,3,6S residue, as well as the carboxylate group in the IdoA2S residue in the pentasaccharide have the strong interaction with the peptide amino acids Lys_{158} , Lys_{159} and Asn_{162}. The aromatic ring of the Phe_{160} residue adopted the position between the N-S group in the reducing end GlcN,6S residue and the carboxylate in the IdoA2S residue. Similarly to the above-mentioned pentasaccharide, the N-sulfate groups in the non-reducing end glucosamine residues, the 6-O-sulfates in the GlcN,3,6S and the carboxylate groups in the IdoA2S residues in structurally-related pentasaccharides showed important role in interactions with the peptide. The NAc groups in the reducing-end GlcNAc residues seem to weaken the interaction with the peptide.

L021
SUGARS & ENZYMES: EXPLORING AND EXPLOITING THE POTENTIAL OF SYNTHETIC GLYCOPROTEINS

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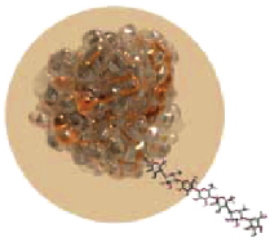
Sugars are critical biological markers that modulate the properties of proteins. Our work studies the interplay of proteins and sugars. This lecture will discuss recent developments our laboratory in two areas: (i) glycoconjugates and their use in potential therapy; and (ii) carbohydrate-processing enzyme mechanism – the engineering and study of glycosidases & glycosyltransferases and the synthesis of probes of their mechanism.



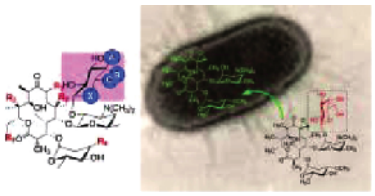
(i) Glycoconjugate synthesis: To explore the key properties of glycosylated macromolecules e.g., proteins, there is a continuing need for methods that not only allow preparation of pure glycosylated proteins, but will also allow the preparation of non-natural variants for e.g. structure-activity relationships (SARs).[1] We have developed methods

for precise protein glycosylation[2] that allowed the effects of protein glycosylation to be explored precisely for the first time.[3] Glycosylated enzymes can be used in • preparative biocatalysis[4] • drug delivery[5] and • selective protein degradation[6].

A new class of glycoconjugate, the glycodendriprotein[7] acts as a powerful nanomolar inhibitor of bacterial interactions.

Newly developed glycoviruses allow targeted gene transfer.[8]

(ii) Carbohydrate-Processing Enzymes: These enzymes are powerful tools not only for use in glycoside synthesis but also as potential targets for therapeutic intervention.



Many elegant studies have made significant advances towards understanding the reactive catalytic mechanisms of these enzymes. However, the mechanism by which substrate specificity is determined is still largely unclear. We have begun to explore the underlying basis of these catalysts[9] and methods to enhance their synthetic utility. For example, through the use of a novel high throughput mass spectrometric screening system[10] we have identified enzymes capable of remodelling macrolide antibiotics to enhance function. Secondly, we have developed methods for the ready construction of arrays of inhibitors as probes of carbohydrate-processing enzymes and which have allowed the identification of novel inhibitors.[11] These include novel stereodynamic aza-sugar strategies that have allowed the first synthesis of naturally occurring hydrophobically-modified aza sugar, Adenophorine[12] & creation of targeted libraries of antivirals.[13]

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L022
RATIONAL DESIGN OF SMALL MOLECULE GLYCOMIMETIC COMPOUNDS THAT BIND MULTIPLE EPITOPES

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Rationally designed glycomimetics based on the bioactive conformation of functional carbohydrates have the potential to contain enhanced activity for the target molecules while possessing the desired pharmacological properties of small molecule drugs. In some well known cases, the native carbohydrate interactions are relatively weak. By exploring secondary sites adjacent to the carbohydrate binding domain, glycomimetic compounds can be designed with enhanced binding activities and act as potent inhibitors of the native carbohydrate interaction. A classic example is the binding of E, P, and L selectins to sialyl $Le^{a/x}$ epitopes. Over 5 generations of rationally designed compounds resulted in glycomimetics with high activity for the carbohydrate binding domain. We explored a secondary site on P selectin that binds sulfated moieties by screening a library of sulfated building blocks for drug development. Heterobifunctional molecules were designed to incorporate both binding domains, thereby binding with high affinity to all three selectins. Inhibitory activity for P-selectin for 2 such glycomimetics (GMI-1010, GMI-1011) improved 20 to 30 fold. In intravital microscopy assays in normal Swiss albino mice, GMI-1010 and GMI-1011 immediately inhibited cell adhesion in vivo that progressed in intensity throughout the 30 minute observation period. GMI-1010 and GMI-1011 were also tested in mouse models for effects on leukocyte migration to a site of inflammation in an air pouch model. Different dosing regimens showed statistically significant inhibition of cell migration between 2 to 10 mg/kg. In a second example, rationally designed glycomimetics are potent inhibitors of PA-IL and PA-IIL lectins of *Pseudomonas aeruginosa* and have potential for therapy of antibiotic-resistant infection.

L023
ENZYMATIC RECONSTRUCTION OF GLYCOSAMINOGLYCANS

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Proteoglycans are complex glycoconjugates that are composed of a core protein and glycosaminoglycan chains. The endo-type glycosidases were investigated with the aim of performing enzymatic synthesis of proteoglycans or glycosaminoglycans. In addition to their main hydrolysis reaction, it is known that many glycosidases catalyze transglycosylation reaction as a reverse reaction. We investigated the transglycosylation mechanism of testicular hyaluronidase, which is an endo-β-*N*-acetylhexosaminidase. When hyaluronic acid, longer than hexasaccharides and having a GlcUA at the nonreducing terminal, was used as a substrate, this enzyme released the disaccharides whose structure was GlcUAβ1-3GlcNAcβ1 from the nonreducing terminal of hyaluronic acid chains by hydrolysis activity.

Simultaneously with this hydrolysis reaction, this enzyme could successively transfer the released disaccharides to the nonreducing terminal of the acceptor hyaluronic acid. The testicular hyaluronidase also acted on the GalNAcβ1-4GlcUA structure in chondroitin (Ch), chondroitin 4-sulfate (Ch4S), chondroitin 6-sulfate (Ch6S), and other glycosaminoglycans, as well as hyaluronic acid. Therefore, by repeating the transglycosylation using suitable combinations of Ch, Ch4S, Ch6S, and other glycosaminoglycans as acceptors and donors, we succeeded in custom-synthesizing new hybrid glycosaminoglycans. A library consisting of more than 100 octa- and decasaccharides has been custom-synthesized to date using this enzymatic reconstruction method. These oligosaccharides will be useful as models to elucidate relationships between structure and functions of glycosaminoglycan oligosaccharides. We will also introduce a study on the glycosaminoglycan structure necessary for adhesion of *Plasmodium falciparum*-infected red blood cells to placenta as an example of a biological study using custom-synthesized chondroitin sulfate dodecasaccharides.

L024
INFLUENZA: NEXT TARGET IS HEMAGGLUTININ

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Neuraminidase inhibitors were surely predominant during the last decade of the struggle against influenza virus. Being a notable example of objectified drug design, these substances, however, have not solved in practical sense the problem of therapy or prophylaxis of this disease. Since 1997, when the first cases of human infection with avian H5N1 viruses were documented, the treat of new influenza pandemy hanged over mankind. Really dangerous is, that despite intense therapy most part of humans infected with H5N1 virus died. Obviously, principally new approaches are necessary. We have demonstrated in *in vivo* experiments that low doses of multivalent 6'SLN derivatives cure influenza; moreover, they can be used ih prophylactic regimen. Two classes of multimerics are studied as therapeutics. The first one includes oligomeric molecules where trisaccharides 6'SLN are situated at optimal distances. The second one is glycopeptides capable of assembling or to virus-promoted assembling. In the latter case glycopeptide remains monomeric before the meeting with virus and is assembled to multimer only on the virion surface.
N.V.Bovin, A.B.Tuzikov, A.A.Chinarev, A.S.Gambaryan. Multimeric glycotherapeutics: new paradigm. *Glycoconjugate J.*, **21**, 471-478 (2004).

L025
INHIBITION OF THE INFLUENZA VIRUS INFECTION BY GLYCOMIMETIC PEPTIDES SELECTED FROM A PHAGE LIBRARY

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Influenza hemagglutinin (HA) is known to bind sialylgalactoside having α2-3- or α2-6- linkages on host cells. It has been reported that several glycopolymers having sialic acids inhibited the influenza virus infection. In the present study, we developed glycomimetic peptides as inhibitors that bind to the receptor-binding pocket of HA.

In order to obtain peptides that are mimics of sialyl oligosaccharide (sialyllactose or sialyl Lewis^X), a phage-displayed random pentadecapeptide library was employed. The HA-binding peptides showed the affinity for both HA1 and HA3 subtypes, and inhibited the infection of MDCK cells by influenza virus of H1N1 and H3N2. Although the theoretical molecular diversity of pentadecapeptide is calculated to be $3.3x10^{19}$, the number of phage library employed was only $2.5x10^8$. Therefore, it was considered that the selected peptides were not optimum sequences. In order to obtain the improved peptide sequences, two kinds of sublibraries of the sequence selected by the primary biopanning were prepared using the molecular evolution engineering involving error prone PCR or synthetic oligonucleotide method. Through the affinity selections for HA1 and HA3 using the sublibraries, several peptide sequences having mutations were obtained. Many of the mutant phage clones showed higher binding affinity for both HA1 and HA3 subtypes than the original phage clone did. The evolved peptides inhibited the infection of MDCK cells by influenza virus of H1N1 and H3N2 more efficiently than the original peptide sequence.

L026
NANO"SOLUTIONS" FOR THE DELIVERY OF GLYCOLIPIDS AND SPHINGOLIPIDS

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The era of glycolipid and sphingolipid-based therapeutics is now a reality. Enzyme replacement therapy and substrate reduction therapy is now the standard of care for patients with Gaucher Disease. Glycolipid and sphingolipid mimetics are now being evaluated as immunotherapeutics, cancer chemotherapeutics and anti-virals. Yet, the true success of utilizing glyco- and sphingo-mimetics as therapeutics is often

not realized due to a lack of selective targeting strategies as well as less than optimal solubility and pharmacokinetic profiles. Thus, the use of nano"solutions" to effectively package and target glycol- and sphingo-mimetics is of critical clinical relevance. To that end, we have recently optimized two nanotechnologies to deliver chemotherapeutics, including glycosphingomimetics, to cancerous lesions. The first technology is an 80 nm-sized pegylated cationic liposome that has the capability of simultaneously delivering multiple chemotherapeutics and siRNA in a tumor-targeted modality. As proof of concept for combinatorial drug delivery via nano-liposomes, we have demonstrated that our proprietary liposomal formulations can deliver up to 40 molar percent ceramide as well as multiple siRNAs to *in vitro* and in vivo tumor models, resulting in tumor stabilization and regression. The second technology is a 5 to 20nm-sized calcium phosphosilicate nanoparticulate, known as a Molecular Dot. These Molecular Dots can encapsulate both sphingolipid mimetics and fluorophores to simultaneously treat and image melanoma. In addition, Molecular Dots exhibit less agglomeration, less toxicity, less photobleaching and a continuous emission profile compared to semi-conductor-based nanotechnologies. In conclusion, nanotechnology has the potential to "deliver" the promise of glycolipid-based pharmaceutics.

L027
SIALIDASES: PERFECTLY DESIGNED FOR PURPOSE

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The sialidase superfamily is exquisitely designed for purpose, appearing in a variety of oligomeric forms, and with the common catalytic domain often decorated with additional carbohydrate-binding domains. The active sites of the catalytic domains contain a number of conserved amino acids suggesting a common catalytic mechanism. Our recent studies on the *nanI* sialidase from *Clostridium perfringens* have shown that this may proceed via a covalent intermediate, supporting earlier work on the *Trypanosoma* trans-sialidase. Most sialidase active sites are very rigid, inducing strain in the substrate. In contrast, the active site of the paramyxovirus hemagglutinin-neuraminidase is pliable and serves as both a sialic acid recognition site and a sialic acid hydrolysis site, with conformational changes that appear to be related to the induction of fusion[1].
Sialidases that have additional carbohydrate-binding domains may gain a catalytic advantage over those that do not[2], and probably help target the enzyme to appropriate substrates. Our studies on the *Vibrio cholerae* sialidase have revealed that it contains a sialic acid lectin domain that has an unusually high affinity for sialic acid[3]. The sialidase from *Micromonospora viridifaciens* contains a galactose binding domain, and our recent studies have shown how this may work in concert with the catalytic domain.
Structural studies on a variety of sialidases from viruses, bacteriophage, bacteria, parasites and mammals have shown a remarkable structural repertoire for this superfamily of enzymes.

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L028
PATHOLOGICAL IMPLICATIONS OF UP-REGULATION OF PLASMA MEMBRANE-ASSOCIATED SIALIDASE (NEU3) IN HUMAN CANCER

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Human plasma membrane-associated sialidase (NEU3) has been implicated in many cellular events through modulation of gangliosides. Our previous studies demonstrating a marked increase in NEU3 expression in human colon cancers suggested that NEU3 is involved in cancer cell apoptosis (Kakugawa, Y. et al. Proc. Natl. Acad. Sci. U. S. A. 99, 10718-10723, 2002). To elucidate the molecular mechanism, the gene was silenced by siRNA or overexpressed in human cells. Knocking down NEU3 by the siRNA induced apoptosis with no special stimuli in HeLa cells, as determined by MTT assays and TUNEL assays. Colon adenocarcinoma HT-29 cells seemed to undergo similar changes, but WI-38 fibroblast cells were not significantly affected even under decreased NEU3 mRNA level. In line with these results, caspase 3 was up-regulated in the siRNA-transfected cells, whereas NEU3 overexpression down-regulated the activity. Furthermore, NEU3 knock down decreased Ras activation, and NEU3 overexpression stimulated Ras activation and subsequent enhancement of phosphorylation of ERK and Akt. Ras activation by NEU3 was abrogated by PP2 (src inhibitor) or AG1478 (EGF receptor inhibitor), but not by Go6983 (PKC inhibitor), suggesting that the possible upstream targets might be tyrosine kinases such as src and EGF receptor at cell surface, and the following stimulation of ERK and Akt phosphorylation through Ras activation inhibits cell apoptosis. NEU3 thus is an essential gene for cell survival and the siRNA could have potential utility for the gene-based therapy of human cancers.

L029
PLASMA MEMBRANE GANGLIOSIDE SIALIDASE IN AXONAL DETERMINATION AND REGENERATION.

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2- Miyagi Prefectural Cancer Center, Division of Biochemistry, 47-1 Nodayama, Medeshima-Shiode, Natori, Miyagi, 981-1293 Japan.
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Axon specification triggers the polarization of neurons and occurs following localized changes in actin dynamics. Plasma membrane ganglioside sialidase (PMGS), an enzyme that regulates polysialic gangliosides/GM1 ratio, asymmetrically accumulates at the tip of one neurite of the unpolarized neuron and is found later along the future axon. Suppressing PMGS activity blocks axonal generation whereas stimulating it accelerates the formation of a single axon, not several. PMGS induces axon specification by enriching the local GM1 concentration and, thus, the activity of TrkA receptors, triggering PI3K/Rac1-dependent inhibition of RhoA and the consequent actin depolymerization in one neurite only. Furthermore, PMGS over-expression enhances the regeneration capacity of the axons after sectioning them *in vitro*, the same effect we observed with non-overexpressing neurons treated with recombinant Galectin-1, that specifically binds and cross-links GM1 carbohydrate moieties. Our results unveil a molecular mechanism spatially restricted to the plasma membrane of a single early growth cone, that prompts neuronal polarity by specifying the future axon. In more mature cells, we envision that axons would only maintain their identity after injury, and regenerate, if GM1 accumulates locally over a certain threshold that we produced by PMGS over-expression or by local stabilization by cross-linking.

L030
LYSOSOMAL SIALIDASES: BIOLOGY AND PATHOLOGY.

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Sialidases or neuraminidases are glycohydrolytic enzymes that remove terminal sialic acid residues from sialylated glycoproteins, oligosaccharides and glycolipids. Three different classes of mammalian sialidases: lysosomal (Neu1, gene *NEU1*), cytoplasmic (Neu2, gene *NEU2*) and plasma membrane (Neu3, gene *NEU3*) have been described. These 3 classes of sialidases are different in their substrate specificity, Neu1 being active mostly against oligosaccharides and glycopeptides, whereas Neu2 and Neu3 are also capable of hydrolysis of sialylated gangliosides. Inherited deficiency of Neu1 in humans, due to mutations in the *NEU1* gene causes severe multisystemic neuradegenerative disorder, sialidosis. Clinically similar disorder, galactosialidosis is caused by the secondary Neu1 deficiency due to genetic defects in lysosomal carboxypeptidase, cathepsin A that forms a complex with Neu1 and activates it. Recently a novel lysosomal sialidase, Neu4 equally active against all classes of sialylated glycoconjugates has been described. The sialylated gangliosides (GD1a, GM2, GM3) are hydrolyzed by Neu4 in the presence of small activator proteins saposins and GM2-activator protein that are required for degradation of glycolipids by other lysosomal glycosidases. Using expression vector system, which directs the synthesis of siRNAs specific to Neu4 gene, we generated stable loss-of-function phenotype in HeLa cells. Transfected cells showed large heterogeneous lysosomes containing lamellar structures and vesicular profiles of different sizes indicating that down-regulation of Neu4 expression causes lysosomal storage. Taken together, our results strongly suggest the potential involvement of Neu4 in the lysosomal catabolism of glycolipids.

L031
ENHANCED EXPRESSION OF SIALIDASE ACTIVITY IN ACTIVATED HUMAN LYMPHOCYTES AND MONOCYTE-DERIVED CELLS INFLUENCES CELLULAR ACTIVITY

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Modulation of the sialic acid content of cell surface glycoproteins and glycolipids influences the functional capacity of cells of the immune system. Although four genetically-distinct mammalian sialidases (Neu1-4) have been identified, the expression of only lysosomal Neu1 and plasma membrane-associated Neu3 is increased in activated human T lymphocytes and in monocyte-derived macrophages and dendritic cells. Differentiation of monocytes *in vitro* into macrophages or dendritic cells was associated with up to a 20-fold increase in Neu1 activity. Similarly, activation of lymphocytes resulted in a five-fold increase in Neu1 activity. In contrast, the activity of Neu3 increased no more than two-fold in activated lymphocytes and in monocyte-derived cells. The increases in Neu1 enzyme activity correlated with increased synthesis of Neu1-specific RNA as determined by real time RT-PCR. Although localized predominantly in lysosomes in freshly-isolated cells, Neu1 was detected on the surface of activated lymphocytes and of macrophages and

dendritic cells by flow cytometry and confocal microscopy using Neu1-specific antibodies. Sialidase activity in activated lymphocytes and monocyte-derived cells was associated with the expression of specific cytokines. In activated lymphocytes, IFN-γ expression was decreased when cells were maintained in culture in the presence of competitive sialidase inhibitors or anti-Neu1 or anti-Neu3 antibodies. Similarly, the expression of IL-6 and IL-12p40 was reduced when cell surface sialidase activity of monocytes was inhibited during differentiation into monocyte-derived cells. Thus, the expression of both Neu1 and Neu3 sialidases on the surface of peripheral blood mononuclear cells influences the expression of specific cytokines that control immune responses.

L032
MEMBRANE ASSICIATED SIALIDASES NEU3 AND NEU4: CELLULAR AND BIOCHEMICAL CARACTERIZATION

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Sialidases (E.C.3.2.1.18) are a group of glycohydrolytic enzymes, widely distributed in nature, which cleave sialic acid residues from glycoconjugates. Four genetically distinct forms of mammalian sialidases have been characterized, with different cellular localization (lysosomal, cytosolic and membrane-associated) and substrate specificity. Neu3 and Neu4 are the membrane-associated members of the family and show different cellular and biochemical properties. First of all, *NEU3* gene is ubiquitously expressed whereas *NEU4* transcript is detectable at lower levels and mainly in liver. Second, the proteins have different subcellular localization as demonstrated by confocal microscopy studies. Neu3 shows a typical cell surface distribution and is also localized in intracellular vesicular structures that represent part of the endosomal compartment. On the contrary, the murine form of Neu4 is associated to an intracellular membranous network spreading from the nuclear region to the cell periphery. Third, the two enzymes show different activities toward ganglioside substrates. Neu3 is highly active toward these sphingolipids, as demonstrated by "in vitro" assays and by the modification of the ganglioside pattern detectable in COS7 cells overexpressing the enzyme. Moreover, the protein modifies the ganglioside content of the sphingolipid enriched domains. Conversely, COS7 cell overexpressing Neu4 do not show significant modifications on the ganglioside cell content. Surprisingly, Neu4 transfected cells are able to hydrolyze exogenous GD1a under conditions that prevent lysosomal activity, suggesting a role of "rescue enzyme" for the last member of the mammalian sialidase family.

L033
ENDOSIALIDASE NF – CRYSTAL STRUCTURE AND STRUCTURE-FUNCTION RELATIONSHIPS OF A POLYSIALIC ACID BINDING AND DEGRADING ENZYME

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Polysialic acid (polySia) is as posttranslational modification of the neural cell adhesion molecule (NCAM) crucially involved in neural development and plasticity. Endosialidases associated with phages that infect polySia encapsulated bacteria are the only known enzymes that specifically hydrolyse polySia and are therefore widely applied in polySia related neurosciences and cancer research.
We have solved the first crystal structure of an endosialidase (endoNF). The enzyme assembles into an SDS-resistant homotrimer with mushroom like outline that is stabilized by an unusual triple-intertwined stalk domain. The unique polySia-specificity evolved from functional combination of three distinct domains: a β-barrel domain, a 6-bladed β-propeller typical for exosialidases and a triple β-helix characteristic for bacteriophage tailspike proteins. Cocrystals with oligomeric sialic acid allowed the identification of polySia binding sites in the β-barrel and the triple-intertwined stalk domain of endoNF. To identify residues that are essential for the endosialidase reaction, a panel of mutants was generated by site-directed mutagenesis. Mutants were purified, analyzed for activity, polySia-binding and trimer stability. Several residues were detected, that are required for either cleavage or binding of polySia. In addition, mutations were found that drastically reduce the stability of the homotrimeric endoNF-complex. In conclusion, the reaction mechanism of endoNF appears to be different from that described for exosialidases. The active site differs markedly since highly conserved residues believed to be key-residues of the exosialidase reaction are not preserved in endoNF. Trimerization is essential for endoNF activity. From our studies we hypothesize, that the catalytic trimer simultaneously binds and degrades three polySia chains.

L034
CHARACTERIZATION OF THE C-TERMINAL ASSEMBLY DOMAIN OF ENDOSIALIDASES

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Endosialidases are tail spike proteins of bacteriophages infecting *Escherichia coli* K1. By degrading the thick polysialic acid capsule of the host they play a key role in the infection cycle. All endosialidases characterized so far share a modular architecture. The central catalytic part is flanked by a variable N-terminal and a short C-terminal domain which is released by proteolytic cleavage at a highly conserved serine residue. The intact C-terminal domain is essential for assembling the catalytic homo-trimer but is dispensable in the mature enzyme [1].

Using circular dichroism (CD) and gel filtration we analysed the secondary and quaternary structure of the C-terminal domain of the endosialidase of bacteriophage K1F (endoNF). The separately expressed C-terminal domain shows identical CD spectra and retention volumes as the C-terminal domain isolated after proteolytic processing, demonstrating that it folds as an independent domain. In contrast to the beta-fold rich catalytic part of endoNF [2], the C-terminal domain was found predominantly alpha-helical. Interestingly, we identified homologous C-terminal domains also in proteins lacking endosialidase activity. These include the L-shaped tail fibre protein of Coliphage T5, the neck appendage protein gp12 of Bacillusphage GA-1, and the K5 eliminase of an *E. coli* K5 prophage. We demonstrate that in all three proteins the C-terminal domain is cleaved off at the highly conserved serine residue, and non-cleavable proteins were generated by exchanging this residue to alanine. Our data indicate that the C-terminal domain acts as a general assembly domain required for the folding of several phage proteins.

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L035
THE GLYCOBIOLOGY OF INFLUENZA VIRUSES

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Relative ability of hemagglutinin (HA) to recognize the preferred receptor species of human influenza viruses - Neu5Ac2-6Gal in contrast to the avian virus receptor, Neu5Ac2-3Gal – affects its ability to infect humans and cause disease (1). The 1918 pandemic influenza A strain is thought to have originated from an avian source. We produced virus that contains the viral HA gene of the 1918 strain, and found that the virus preferentially binds Neu5Ac2-6Gal (2), indicating that 1918 strain must have circulated in humans or possibly another adaptive host such as pigs with human-type receptors to develop a preference for the human sialyl sugar chain receptors. Since 1997, highly pathogenic H5N1 influenza A viruses have been directly transmitted from birds to humans. In 2003, H5N1 (A/HK/213/03) viruses were isolated from the family members. Both Siaα2-3Gal and Siaα2-6Gal were detected in the primary culture of the human tracheal cells. Receptor binding assay of A/HK/213 showed its capacity to recognize both the α(2-3)- and α(2-6)-linked sialic acids (3), suggesting that after its transmission from a bird to a human, it may have acquired the ability to recognize Siaα2-6Gal. These results suggest that highly pathogenic avian H5N1 viruses can be transmitted to humans if exposed in high concentration, and may have an ability to infect from human to human after the mutation of the receptor binding specificity.

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L036
INFLUENZA RECEPTOR RECOGNITION

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ABSTRACT NOT AVAILABLE

L037
A NOVEL CLASS OF HIGH-AFFINITY INHIBITORS OF TYPE 1 FIMBRIAL ADHERENCE OF *ESCHERICHIA COLI* DISCLOSED IN THE CRYSTAL STRUCTURE OF THE FIMH ADHESIN

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Introduction Mannose-binding type 1 pili are important virulence factors for the establishment of Escherichia coli urinary tract infections. These infections are initiated by adhesion of uropathogenic E. coli to uroplakin receptors in the uroepithelium via the FimH adhesin located at the tips of type 1 pili. Blocking of bacterial adhesion allows to prevent the infection.

Experimental approaches We provide a 1.7 Å resolution crystal structure of FimH in complex with butyl α-D-mannoside and present equilibrium binding data with mannose, common mono- and disaccharides, and a series of alkyl and aryl mannosides.

Results The crystal structure illustrates that the lectin domain of the FimH adhesin is a stable and functional entity and that an exogenous butyl α-D-mannoside, bound in the crystal structure, exhibits a significantly better affinity for FimH (K_d = 0.15 μM) than mannose (K_d= 2.3 μM). Exploration of the binding affinities of α-D-mannosides with longer alkyl tails revealed affinities up to 5 nM [1]. Aryl mannosides can also bind with high affinities and their relative affinities for the FimH adhesin correlate very well with the relative concentrations of the same glycans required for the inhibition of adherence of type-1 piliated E. coli [2].
Conclusions New anti-adhesive drugs to prevent anticipated and recurrent infections could be based on alkyl mannosides. Those allow specific interactions targeted to the urinary tract. With their nanomolar affinities a significant obstacle of low affinity for carbohydrate-based drugs is overcome. Moreover, anti-adhesives aim to expell, not kill, bacteria, and avert antibiotics resistance.

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L038
THE DIVERSE SPECIFICITY OF HELICOBACTER PYLORI

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The human gastric pathogen Helicobacter pylori is an etiologic agent of chronic active gastritis, peptic ulcer and gastric adenocarcinoma. A prominent feature of the H. pylori-induced gastritis is infiltration of neutrophils into the gastric epithelium. Neutrophils play a major role in epithelium injury, since these cells have toxic effects on the epithelial cells by releasing reactive oxygen and nitrogen species and proteases. An additional virulence factor of H. pylori bacterial cells is thus the neutrophil activating capacity, i.e. the ability of certain H. pylori strains to activate human neutrophils in the absence of opsonins. Several putative receptors of H. pylori have been demonstrated. Thus, the binding of the bacterium to such diverse compounds as phosphatidylethanolamine, gangliotetraosylceramide, the Leb blood group determinant, heparan sulphate, the GM3 ganglioside, sulfatide, lactosylceramide, lactotetraosylceramide and neolacto-carrying glycoconjugates has been reported. Still, only three H. pylori carbohydrate-binding proteins, the Leb -binding BabA adhesin, the sialic acid-binding SabA adhesin and the sialyl-neolacto-binding neutrophilactivating protein HP-NAP, have been fully characterized. The structural requirements for ganglioside recognition by the SabA adhesin and HP-NAP have been determined using a collection of complex gangliosides. The relative contribution of the SAbA adhesin and the neutrophil-activating protein HP-NAP to the activation of human neutrophils in terms of adherence, phagocytosis and oxidative burst has also been investigated, demonstrating that SabA-mediated binding of H. pylori to sialylated neutrophil receptors plays a pivotal role in the neutrophil activation, and thus is a virulence factor important for the pathogenesis of H. pylori infection.

L039
GLOBOTRIAOSYL CERAMIDE RAFTS IN MICROBIAL PATHOLOGY.

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The glycosphingolipid, globotriaosyl ceramide (Gb_3) is the functional receptor for the *E. coli* derived family of verotoxins, which cause the renal pathology of hemolytic uremic syndrome, and is involved in the host cell binding and membrane fusion of HIV. In both these receptor functions, the accumulation of Gb_3 within plasma membrane lipid rafts plays a central role. VT1 binding is preferentially associated with plasma membrane lipid rafts as determined by detergent extraction/sucrose gradient centrifugation, whereas VT2 is primarily associated with the non-raft Gb_3 fraction. Separate and colocalized VT1 and VT2 binding sites on the cell surface were monitored during toxin internalization/retrograde trafficking, indicating distinct Gb_3 pools within the membrane. We have synthesized a water-soluble Gb_3 mimic by substituting the fatty acid moiety with an adamantan frame. This compound retains receptor activity of membrane Gb_3 in solution. It also is an apparent mimic of lipid raft Gb_3 since it behaves similarly to Gb_3/cholesterol in HIV gp120 monolayer binding and competes more effectively for VT1 than VT2 cell Gb_3 binding. AdamantylGb_3 is an effective inhibitor of both X5 and X4 HIV infection and is an inhibitor of gp120/CD4/ chemokine receptor mediated membrane fusion.
Since Gb_3 receptor function for VT1 and gp120 is largely lipid raft dependent, we have developed a sucrose gradient assay to address the biochemical properties necessary for Gb_3 lipid raft binding. Significant differences in the cholesterol and sphingomyelin content required to optimize VT1 and VT2 Gb_3 raft binding were found. Gb_3 fatty acid homologues were made and remarkable differences in the ability of such Gb_3 fatty acid isoforms to form cholesterol complexes capable of binding VT1 were observed. Isoforms unable to form rafts recognized by VT1 could have a dominant negative effect in the presence of other Gb_3 fatty acid isoforms. Even for isoforms which show strong VT1 binding, the majority of the Gb_3/cholesterol complex (> 90%) is separated from the bound complex. Fatty acid chain length had no effect on VT2-Gb_3 raft binding. These studies reveal an unsuspected complexity in the presentation of GSL carbohydrate within lipid rafts.

L040
COLLECTINS IN DEFENCE AGAINST INFECTIOUS DISEASES

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The collectins constitute a small subfamily of animal lectins defined as molecules having a C-type carbohydrate recognition domain combined with a collagen-like region. The polypeptide gene products organise themselves into triplets having a long intertwined tail combined with a cluster of globular heads, giving a 3-dimensional structure loosely resembling C1q. Higher oligomers may assemble from the basic triplet subunits. Eight members of this subfamily so defined have been described: mannan-binding lectin (MBL); surfactant protein A (SP-A); surfactant protein D (SP-D), and closely related molecules found only in the *Bovidae*, conglutinin, CL-43 and CL-46; CL-L1, an intracellular collectin; and CL-P1 (SRCL), a scavenger receptor found on the surface of endothelial cells, with a rather more complex structure than the others. All the collectins are thought to function in host defence against infectious pathogens as pattern recognition molecules, although other functions may co-exist. For the bovine collectins, the evidence is mainly circumstantial. For SP-A and SP-D, the creation of knockout mice has been helpful. For MBL, knockout mice have provided supplementary data to the large literature on disease associations of human MBL deficiency/insufficiency. MBL is the only collectin known to activate the complement system; however, L-ficolin and possibly H-ficolin, members of a distinct lectin subfamily also characterized by a collagen-like domain, can also activate complement by a similar mechanism using MBL-associated serine protease-2 to generate a C3 convertase.

L041
WHEN THE CLINICIAN MEETS THE SCIENTIST: BEYOND THE STORAGE EFFECTS

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The glycosphingolipid lysosomal storage diseases are a group of monogenic human disorders caused by the impaired catalytic activity of enzymes responsible for glycosphingolipid catabolism. Clinical presentation of the diseases is heterogeneous, with little obvious correlation between the kind of accumulating glycosphingolipid and the disease progression or pathogenesis. Fabry disease, an X-linked sphingolipidosis (SL), is one of the recently treatable SL by enzyme replacement therapy (ERT). The affected patients present a deficient alpha galactosidase activity which results in the progressive accumulation of globotriaosylceramide (GL3) and other glycosphingolipids in tissue lysosomes throughout the body. In classically affected patients, GSL accumulation in the vascular endothelium eventually culminates in life-threatening renal, cardiac, and cerebrovascular disease. Clinicians discovered recently that women as well as children might be affected. New issues have come up such as what is the best time to begin with treatment? When should we begin to treat women? What is the optimal dose for women? What is the best way to evaluate the efficacy of the treatment? Could we treat the patients with a cheaper and more effective treatment? Is there a way to really treat the patients and not only to change their phenotype?
Additional insights into the cell dysfunction mechanism might give some of the answers and open the door to new more effective treatments.

L042
HUMAN GM3 SYNTHASE DEFICIENCY CAUSES AN INFANTILE ONSET EPILEPSY SYNDROME

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Although the relationship between defects in ganglioside catabolism and lysosomal storage diseases is well documented, there have been no proven reports of defects in ganglioside biosynthesis associated with human disease.
In this study, we have identified a severe human epilepsy syndrome caused by the loss of GM3 synthase function. This disorder is inherited as an autosmal recessive trait and results in an infantile onset symptomatic epilepsy syndrome associated with developmental stagnation and blindness. We have studied 8 affected individuals with this syndrome in two families from a large Old Order Amish pedigree. Gene sequencing identified a nonsense mutation in the SIAT9 gene, which is predicted to result in the premature termination of the GM3 synthase enzyme (CMP-NeuAc: Lactosylceramide α-2,3 sialyltransferase, EC 2.4.99.9). GM3 synthase is a sialyltransferase that catalyses the initial step in the biosynthesis of the majority of complex ganglioside species from lactosylceramide (LacCer). Biochemical analysis of plasma glycosphingolipids (GSLs) confirmed a lack of GM3 synthase activity in all the affected individuals. There was a complete absence of GM3 ganglioside and its biosynthetic derivatives and a concomitant increase in LacCer and its alternative neutral GSL derivatives, Gb3 and Gb4. These data suggest that a lack of complex gangliosides and/or the accumulation of precursors of ganglioside synthesis result in neuronal instability in the CNS. Elucidating the mechanism(s) through which this disease phenotype develops will shed light on ganglioside functions in the brain.

L043
G_{M1}-GANGLIOSIDE AS APOPTOTIC SIGNAL IN NEURODEGENERATIVE G_{M1}-GANGLIOSIDOSIS

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G_{M1}-ganglioside (G_{M1}) is a major sialoglycolipid of neuronal membranes that, among other functions, modulates calcium homeostasis. Progressive accumulation of G_{M1} due to deficiency of lysosomal β-galactosidase (β-gal) characterizes the neurodegenerative disease G_{M1}-gangliosidosis. Children with this disorder suffer from a generalized CNS involvement, which results in profound neurological deterioration, mental retardation and early death. The murine model of G_{M1}-gangliosidosis closely resembles the early-onset form of the disease. β-gal$^{-/-}$ mice develop a severe CNS condition, which is associated with massive, age-dependent accumulation of G_{M1} and is accompanied by gradual deterioration of motor functions. We have investigated whether the abnormal intracellular concentration of G_{M1} could be directly responsible for the CNS pathogenesis in the mouse model. The activation of the unfolded protein response (UPR) was tested as a putative cause of neurodegeneration. We demonstrate that in β-gal$^{-/-}$ spinal neurons activation of the UPR leads to the up-regulation of the ER chaperone BiP and the proapoptotic transcription factor CHOP, followed by the activation of the kinase JNK2 and the ER resident caspase-12. The combined induction of these effectors culminates with neuronal apoptosis. G_{M1} -loading of wild-type neurospheres activated this pathway by depleting calcium from the ER and induced cell death. The activation of ER stress-regulated apoptotic pathways did not occur in mice deficient for both β-gal and ganglioside synthase, β-gal$^{-/-}$/GalNAcT$^{-/-}$, which do not accumulate G_{M1}. These findings point to a novel mechanism of neuronal apoptosis, and have significant implications for therapy of this disorder (Supported partly by NIH grant DK52025).

L044
BICISTRONIC LENTIVIRAL VECTOR CORRECTS BETA-HEXOSAMINIDASE DEFICIENCY IN TRANSDUCED AND CROSS-CORRECTED HUMAN SANDHOFF FIBROBLASTS

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Sandhoff disease is an autosomal recessive neurodegenerative disorder characterized by the intralysosomal accumulation of GM2 ganglioside. It is due to mutations in the HEXB gene encoding the hexosaminidases β-chain and results in a hexosaminidases A (αβ) and B (ββ) deficiency. This neurolipidosis, characterized by a predominant involvement of the central nervous system, has a dramatic evolution leading to early death. No efficient treatment is available to date for this disease.
In order to test the feasibility of gene transfer methods in this model, bicistronic lentiviral vectors (SIV) containing the HEXA and HEXB cDNAs under the control of the CMV promoter were constructed. Functional tests of these constructs were performed on deficient fibroblasts from Sandhoff patients. A massive restoration of β-hexosaminidases activity was obtained on synthetic substrates using the bicistronic SIV.ASB vector by comparison with the monocistronic SIV.HEXB vector. The same activity determined on the GM2 natural substrate reached 20% of the normal level in cells transduced with the bicistronic vector. Metabolic labelling experiments showed a large reduction of ganglioside accumulation in SIV.ASB transduced cells, demonstrating that the recombinant enzymes were correctly processed and targeted to the lysosomes. Moreover, enzymes secreted by transduced Sandhoff fibroblasts were endocytosed in deficient cells via the mannose 6-phosphate system, allowing the restoration of the GM2 metabolism in cross-corrected cells. Therefore, our bicistronic lentiviral vector supplying both α and β subunits of β-hexosaminidases may provide a potentially therapeutic tool for the treatment of Sandhoff disease and will now be tested in vivo in hexb-/- mice.

L045
METABOLIC CORRECTION IN THE CNS CELL LINES DERIVED FROM SANDHOFF DISEASE MODEL MICE

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Sandhoff disease is a lysosomal storage disease caused by simultaneous deficiencies of beta-hexosaminidase A (HexA; *αβ*) and B (HexB; *ββ*), due to a primary defect of the beta-subunit gene (*HEXB*) associated with excessive accumulation of GM2 ganglioside (GM2) and oligosaccharides with N-acetylhexosamine residues at their non-reducing termini (GlcNAc-oligosaccharides), and with neurosomatic manifestations. To develop and evaluate the novel therapy for

this disease, we established the central nervous system (CNS) cell lines, including microglia, glial precursor-like cells (GPC) and pro-oligodendrocytic cells (pOL), derived from the neonatal brain of Sandhoff disease model mice (SD mice), which were produced by specific disruption of the *Hexb* gene encoding the murine Hex beta-subunit. We characterized the differentiation markers and demonstrated that these three cell lines expressed Iba-1, A2B5 and O4 antigens, respectively. The cell lines derived from SD mice exhibited the intracellular accumulation of GM2 and GlcNAc-oligosaccharides on immunofluorescence and immunoblotting with monoclonal antibodies specific for the natural substrates. We produced not only the recombinant lentiviral vector encoding the *Hexb* gene but also the recombinant human HexA protein isolated from the conditioned medium of the CHO cell line stably expressing the human Hex alpha -subunit (*HEXA*) and *HEXB* genes. Therapeutic effects of the recombinant lentivirus as well as the human HexA on the CNS cell lines were demonstrated to correct the intracellularly accumulated GM2 and GlcNAc-oligosaccharides. The administered recombinant human HexA were incorporated by the microglia, GPC and pOL cells via the specific glycoreceptors (lectins), including mannose receptor and/or cation-independent mannose-6-phosphate/insulin-like growth factor II receptor.

L046
IMPROVED ANIMAL MODELS OF METACHROMATIC LEUKODYSTROPHY

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Metachromatic leukodystrophy (MLD) is a severe lysosomal storage disorder caused by deficiency in the sulfatide degrading enzyme arylsulfatase A (ASA). Inability to degrade sulfatide causes progressive demyelination and various neurological symptoms. ASA deficient ASA(-/-) mice have been generated as a mouse model for MLD. ASA(-/-) mice store sulfatide in brain, kidney and other organs, as human MLD patients, however, they did not exhibit the progressive demyelination, observed in humans. Taken together these mice correspond to the early stage of MLD. In order to increase sulfatide storage and thereby to generate mouse models resembling the later stages of MLD we generated transgenic mice overexpressing the enzymes catalyzing the synthesis of sulfatide: UDP-galactose:ceramide galactosyltransferase (CGT) and 3'-phosphoadenosine 5'-phosphosulfate:cerebroside sulfotransferase (CST) in neurons and oligodendrocytes, respectively. Sulfatide increased significantly in mice overexpressing CGT in neurons and CST in oligodendrocytes, respectively. This was accompanied by more pronounced behavioral deficits when compared to non-transgenic ASA(-/-) mice. Elevated sulfatide levels in neurons resulted in axonal degeneration. Importantly, increased sulfatide storage in Schwann cells led to demyelination of axons in peripheral nerves, never observed in the non-transgenic ASA(-/-) mice. As in human MLD patients, seizures were observed in transgenic ASA(-/-) mice. Our observations clearly show that the milder phenotype of ASA(-/-) mice when compared to human MLD patients can be improved by increasing the synthesis of sulfatide. These transgenic mice will further improve our understanding of the pathology of the disease and the mechanism leading to demyelination in MLD.

L047
PRINCIPLES OF LIPID RAFT FORMATION: ROLE OF LIPID STRUCTURE

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Lipid rafts are sphingolipid and cholesterol rich domains believed to co-exist in cell membranes with domains rich in phospholipids having unsaturated acyl chains. Rafts are likely to exist in the tightly packed liquid ordered state, while domains rich in unsaturated phospholipids are likely to form the more loosely packed disordered liquid state. The structure of each membrane lipid influences raft stability. For sphingolipids, the stability of tight packing and raft stabilizing abilities was found to decrease in the order ceramide>cerebroside>sphingomyelin. Sterol structure can also affect raft formation. It was found that early biosynthetic precursors of cholesterol pack less well than cholesterol, and do not stabilize rafts strongly, while some late precursors pack better than cholesterol, and stabilize rafts to a greater degree. Interestingly, it was also found that under some conditions sphingolipid and sterol compete for association with rafts such that ordered domains become sterol-depleted. This appears to be due to a limited capacity of rafts for lipids with small headgroups, and differences in the strength of sphingolipid-sphingolipid vs. sphingolipid-sterol interaction. Sterols that stabilize rafts most strongly are most resistant to displacement from rafts. Finally, unsaturated phospholipid acyl chain and headgroup structure can also enhance raft stability. Interestingly, this stabilization was not always found to parallel the ability of unsaturated phospholipids to pack tightly. Instead, some unsaturated lipids that strongly resist formation of tightly packed lipid domains appeared to enhance ordered domain formation. This probably reflects a poor ability to mix with sphingolipids and sterols located in the same bilayer.

L048
GLYCOSPHINGOLIPID-STEROL MODEL MEMBRANES: MODULATION OF LATERAL ELASTICITY BY LIPID STRUCTURE

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Membrane micodomains, i.e. rafts and caveolae, reportedly are enriched in glycosphingolipids (GSLs) and cholesterol, display liquid-ordered phase properties, and function as organizing sites for proteins with certain lipid anchors. We have focused on: 1) identifying the essential structural features that might enable GSLs and cholesterol to form microdomains; 2) defining the physical nature of the membrane environment within GSL-sterol rafts and responsible for their functionality as preferred localization sites for lipid-anchored proteins. To address these issues, we introduced well-defined structural variation into GSLs, using lipid synthetic approaches, and assessed the impact on the physical environment of GSL-sterol mixtures using monolayer model membrane systems. A unique aspect of our monolayer studies has been the discovery that the surface compressional moduli, which reflects lipid lateral packing elasticity, provides a sensitive and reproducible way to gain quantitative insights over a wide range of surface pressures and sterol mole fractions, including those relevant to biomembrane conditions. Application of the preceding strategy to lipid mixtures comprised of cholesterol and simple GSLs (e.g., GalCer and LacCer) has revealed correlations with resistance to solubilization by Triton X-100, a widely used approach to harvest rafts from cells. Our data show that sphingolipid headgroups, interfacial function groups and acyl chain compositions all play roles in modulating changes in the lateral packing elasticity of sphingolipid-cholesterol phases. [Support: NIH/NIGMS 45928 & Hormel Foundation].

L049
BINDING OF DIFFERENT LIGANDS TO SIALYL-TRANSFERASES AND GTB REVEAL COMMON FEATURES OF THESE GLYCOSYLTRANSFERASES

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Glycostructures on eukaryotic cell surfaces play an important role in development and regeneration as well as in the pathogenesis of diseases. The terminal sugars found on these glycostructures, such as the sialic acids, are crucial in the control of many biological processes. The transfer of these sugars to glycoconjugates is controlled by specific glycosyltransferases.

Using saturation transfer difference nuclear magnetic resonance (STD-NMR) methods [1] it is possible to obtain information regarding protein-ligand interactions [2]. These experiments not only allow the detection of binding, but also, the determination of the binding epitope for each ligand at an atomic resolution. The binding epitope of CMP-Neu5Ac with different mammalian and bacterial sialyltransferases reveals that the nucleotide and sugar moiety are both in intimate contact with the protein surface. Further, a fragment based approach revealed that cytosine is the smallest fragment recognised by the enzymes.
An equivalent study conducted on the human blood group B galactosyltransferase (GTB) reveals that in this case uracil is the minimal recognition fragment and that the binding is essentially controlled by the base, with the sugar recognition being only involved in the determination of reaction specificity. This is illustrated by the fact that UDP, UDP-Glc and the natural substrate UDP-Gal all have the same binding affinity.
On the basis of these data, new insights into the structure and function of the active centers of sialyltransferases and GTB will be presented. In particular, the identification of common features and differences between these enzymes will be discussed.

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L050
SURFACE STRUCTURING OF GANGLIOSIDES IN MODEL MEMBRANES.

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The ability of gangliosides to organize themselves on surfaces, even of very simple model system, where they display collective behaviours is well known. Also the influence of the surface packing on the hydrophobic chains arrangement is known. Recent experimental structural results (Wide Angle X-Ray Scattering) suggest that the strong organization of ganglioside headgroups includes 3-dimensional ordering within the hydrophilic layer of the aggregated structure, arising from a complex network of interactions including steric, charge, H-bond contributions. This surface structuring of gangliosides is able to induce a liquid-ordered phase in the hydrophobic region, allowing for the contemporary presence of surface order and core fluidity. In the meanwhile, gangliosides are able to modulate the extent of water structuring in their environment. Recent structural (Raman Scattering) and dynamic (Depolarised Rayleigh Scattering) results suggest that gangliosides can either reduce or reinforce water structuring, with respect to bulk water, according to their surface organization. The gangliosides contribution to the structural and dynamic properties of the domains where they are embedded is then seen to be a multi-level action (core,

surface, environment). While the original aspects of this contribution can be recognized, also the synergic role they can play together with the other typical lipid components of rafts can be imagined, both providing intriguing suggestions for their structure-function correlation.

L051
FORMATION OF STEROL-RICH DOMAINS IN GLYCOSPHINGOLIPID CONTAINING BILAYERS

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Rafts enriched in sphingolipids and cholesterol in the plasma membranes of eukaryotic cells have been implicated to take part in cellular processes, such as signal transduction, membrane trafficking and protein sorting. The aim of this study was to explore whether some specific glycosphingolipids and sterols co-exist in the same ordered domains in mixed bilayer membranes. The formation of sterol-enriched domains in a fluid bilayer was examined using cholestatrienol as a fluorescent reporter molecule, selective for sterol-rich domains, together with a quencher in the disordered phase. The glycosphingolipids used in this study were galactosyl ceramides with oleic (*N*-O-GalCer), palmitic (*N*-P-GalCer) or myristic (*N*-M-GalCer) acid in the *N*-linked position as well as the corresponding lactosyl ceramides (*N*-O-LacCer, *N*-P-LacCer and *N*-M-LacCer). Our results showed that none of these glycolipids were able to form sterol-enriched domains to any greater extent in the temperature range studied when present as the only sphingolipid in the bilayer. However, when *N*-palmitoyl-sphingomyelin was included in the membranes the melting of sterol-enriched domains with increasing temperature could clearly be detected by the cholestatrienol quenching method. We could also see that mixtures of GalCers and LacCers were more prone to form domains with sterol then when these glycolipids were present on their own. The acyl-chain composition of the glycosphingolipids affected the partitioning of sterol into glycosphingolipid-rich domains and the stability of the sterol-rich domains. In a biologically relevant system, where presumably no single species of glycosphingolipid exists on its own, the glycosphingolipids are most likely forming ordered domains that may be enriched in sterols when such are present.

L052
PHOTOAFFINE GLYCOLIPID ANALOGUES BEARING DIAZOCYCLOPENTADIEN-2-YLCARBONYL GROUP, AND THEIR APPLICATION IN BIOLOGICAL STUDIES

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A series of photoaffine glycolipid analogues, gangliosides and lipophilic glycoconjugates, bearing diazocyclopentadien-2-ylcarbonyl (Dcp) group were prepared for biological studies. Carbene generating Dcp group is of small size, it can be iodinated in one stage at microscale. In addition to earlier described ganglioside GM1 analogues with the Dcp group at the end of long (Dcp-GM1-l) or short (Dcp-GM1-s) acyl chain, another GM1 derivative bearing the label in the polar head (Dcp-GM1-h) was prepared. Activated T-lymphocytes (interleukin-2-dependent murine cell line CTLL-2) were incubated with ^{125}I-Dcp-GM1-l for 40 min and photolysed. Among proteins precipitated with antibodies to all IL-2 receptor subunits, only β-subunit revealed substantial radiolabeling; experiments with ^{125}I-Dcp-GM1-s gave similar results. These data show that ganglioside GM1 interacts selectively only with β-subunit of IL-2 receptor.

In the scope of targeted liposomal drug delivery studies, a set of neoglycolipid probes bearing Dcp label was synthesized. The probes are lipophilic glycoconjugates built on the basis of amphiphilic aglycon made up from a diglyceride residue and polyethylene glycol spacer, with Dcp group attached near carbohydrate moiety by a short linker via alkali labile ester bond; this should facilitate analysis of cross-linking products. Photoaffine neoglycoconjugates containing oligosaccharides Sialyl-Lewis X, A trisaccharide and Sulfate-Lewis A, specific for a number of tumor cells, has been synthesized, as well as a control probe bearing pentaol glucitol, to detect nonspecific binding. Photolysis of M3 (murine melanoma) and human promyeloid HL-60 cells after binding with liposomes bearing ^{125}I-labeled neoglycolipid probes resulted in radiolabeling of several proteins with various molecular masses, the labeling profiles differed for different probes and cells. For M3 cells, four proteins were revealed; on the HL-60 cells, six assumed receptors were found. Comparison of the published data with ours let us suggest that detected receptors are some known lectins of mammalian cells.

[Support: RFBR 00-04-48922 and 03-04-48396, ISTC 2185].

L053
THE ROLE OF FATTY ACID CHAINS OF GLYCOSPHINGOLIPIDS ON GLYCOSIGNALING DOMAIN-MEDIATED NEUTROPHIL FUNCTIONS.

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Glycosphingolipids are membrane miner components which cluster with sphingomyelin and cholesterol in cell membrane to form microdomains/lipid rafts with several kinds of transducer molecules, especially with membrane-anchored

signal transduction molecules such as Src family kinases. Glycosignaling domain (GSD) is a kind of microdomains, in which glycosphingolipids function as adhesion molecules and mediate several kinds of cell functions. However, the connection of glycosphingolipids, located in the external leaflet of the bilayer, with signal transducer molecules, located in a cytoplasmic site of membrane, remains to be obscure.
Lactosylceramide (LacCer; CDw17) has been speculated to be participated in bactericidal action of those cells. Recently, we found that LacCer forms GSD coupled with a Src family kinase Lyn on neutrophil plasma membrane. Interestingly, human promyelocytic leukemia HL-60 cells, which were differentiated into neutrophilic lineage by DMSO-treatment, did not respond to anti-LacCer antibodies. LC-MS^n analysis of LacCer isolated from plasma-membrane microdomain fractions of neutrophils revealed that neutrophil LacCer is mainly composed of C24 and C16 fatty acid-containing LacCer. In contrast, there were quite few amounts of C24 fatty acid-contained LacCer in plasma membrane of DMSO-treated HL-60-cells. C24-LacCer loading gave HL-60 cells the ability to perform functions such as chemotaxis, superoxide generation and phagocytosis. In contrast, the C16-LacCer loaded HL-60 cells did not show any kind of LacCer-mediated cell functions. Taken together, these data suggest that C24 fatty acid chains in LacCer plays an important role in the signal transduction in LacCer-enriched GSD of neutrophils.

L054
RAFTS AS MISSING LINK BETWEEN MULTIDRUG RESISTANCE AND SPHINGOLIPID METABOLISM

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We have shown that two ABC transporters (Pgp/MRP1) are enriched in Lubrol resistant membrane domains in multidrug resistant human cancer cells (2780AD/$HT29^{col}$). This localization is independent of caveolae, since 2780AD cell do not express caveolin-1. Although $HT29^{col}$ cells do express caveolin-1, MRP1 and caveolin-1 were dissociated on the basis of differential solubility in Triton X-100. Long-term screening revealed that, during colchicine-induced acquisition of multidrug resistance in $HT29^{col}$ cells, increases in glucosylceramide occurred concomitantly with up regulation of MRP1 expression. Both MRP1 and glucosylceramide were found enriched in Lubrol resistant membrane domains. Resistance to colchicine was determined by MRP1, while glucosylceramide synthase did not contribute. Here we show that aminophospholipids are relatively enriched in Lubrol resistant membrane domains compared to Triton X-100 resistant membrane domains, while sphingolipids are relatively enriched in the latter. Moreover, Lubrol resistant membrane domains contain more protein and lipid mass. Based on these results, we postulate a model for detergent-insoluble glycosphingolipid enriched membrane domains (DIGs) consisting of a Lubrol insoluble/Triton X-100 insoluble and a Lubrol insoluble/Triton X-100 soluble region. The latter region contains most of the ATP-binding cassette transporters as well as lipids known to be necessary for their efflux activity. When compared to drug-sensitive cells, the DIGs in drug-resistant 27870AD cells differ specifically in sphingolipid composition and not in protein, phospholipid or cholesterol content. Together, these data show that multidrug resistance-associated changes in sphingolipids and ATP-binding cassette transporters both occur in detergent-insoluble glycosphingolipid-enriched membrane domains, but in different regions of these domains.

L055
NON-MDR DRUG-RESISTANT PHENOTYPES IN OVARIAN CARCINOMA CELLS ARE DEFINED BY ALTERATIONS OF THE LIPID MEMBRANE DOMAIN ORGANIZATION.

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Introduction: An extensive remodeling of complex glycosphingolipids occurs in some cell lines resistant to antitumor drugs. Here we report on the contribution of altered cell surface properties correlated with changes in complex sphingolipids to the acquisition of non-MDR drug resistant phenotypes in carcinoma cells.
Experimental approaches. The human ovarian carcinoma A2780/HPR cells, resistant to the synthetic retinoid fenretinide (HPR), were developed from parental cells by incubation with increasing concentrations of HPR. A2780/HPR cells were not cross-resistant to other chemotherapeutics and did not overexpress MDR-related proteins. In cells characterized by different sensitivity to antitumor drugs we studied 1) the sphingolipid patterns and metabolism; 2) the expression levels and association with a detergent resistant membrane fraction of caveolin-1 and integrin signaling molecules.
Results. In A2780/HPR cells, the biosynthetic pathway downstream of ceramide was more active than in the parental sensitive cell line, leading to the expression of higher levels of GM3 and GM2. A2780/HPR cells overexpressed caveolin-1, a membrane adapter which couples the integrin subunit to non receptor tyrosine kinases. Caveolin-1 in A2780/HPR cells was mainly recovered in a detergent-resistant ganglioside-enriched membrane fraction. Respect to sensitive cells, A2780/HPR cells showed reduced proliferation rate, and altered adhesion

properties, together with altered expression levels and associations with the detergent-resistant membrane fraction of integrins and intermediate and substrates of integrins signaling.
Conclusions. These results indicated that resistance to HPR is characterized by deep alterations of a lipid membrane domain organization involving a glycosynapse-like signaling complex between GM3, caveolin-1 and members of the integrin signaling cassette.

L056
SPATIAL AND FUNCTIONAL HETEROGENEITY OF SPHINGOLIPID-RICH MEMBRANE DOMAINS

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Little is known about the organization of lipids in biomembranes. Lipid rafts are defined as sphingolipid- and cholesterol- rich clusters in the membrane. Details of the lipid distribution of lipid rafts are not well characterized, mainly because of a lack of appropriate probes. Ganglioside GM1-specific protein, cholera toxin, has long been the only lipid probe of lipid rafts. Recently it was shown that earthworm toxin, lysenin, specifically recognizes sphingomyelin-rich membrane domains. Binding of lysenin to sphingomyelin is accompanied by the oligomerization of the toxin that leads to pore formation in the target membrane. In this study, we generated a truncated lysenin mutant that does not oligomerize and thus is non-toxic. Using this mutant lysenin, we showed that plasma membrane sphingomyelin-rich domains are spatially distinct from ganglioside GM1-rich membrane domains in Jurkat T cells. Like T-cell receptor (TCR) activation and cross-linking of GM1, cross-linking of sphingomyelin induces calcium influx and ERK phosphorylation in the cell. However, unlike CD3 or GM1, cross-linking of sphingomyelin did not induce significant protein tyrosine phosphorylation. Combination of lysenin and sphingomyelinase treatment suggested the involvement of G-protein coupled receptor in sphingomyelin-mediated signal transduction. These results thus suggest that the sphingomyelin-rich domain provides a functional signal cascade platform that is distinct from those provided by TCR or GM1. Our study therefore elucidates the spatial and functional heterogeneity of lipid rafts.

L057
CDG SYNDROMES - CLINICAL PHENOTYPES, CURRENT THERAPEUTIC STRATEGIES AND UPCOMING NEW DISORDERS

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Congenital disorders of glycosylation have become a major group of inherited metabolic diseases during the last decade. More than 15 different disorders are known today. N- and/or O-glycosylation as well as C-mannosylation or GPI anchor biosynthesis can be affected. Accordingly, most CDG patients present with a severe multisystem disease often leading to death early in life. Understanding the molecular bases has been crucial for the development of the two known therapies. The presentation will give an overview over the clinical phenotypes, frequency of the disorders and the current options for spefcific treatment.
Dolichol kinase deficiency is a recently discovered glycosylation disorder and will be presented in more detail. It is the first disorder to affect the biosynthesis of the dolichol phosphate carrier molecule. Affected patients show a very severe disease with loss of hair, ichtyosis of the skin, dilative cardiomyopathy, severe muscular hypotonia and death in infancy. 4 patients have been discovered so far. Due to the reduced biosynthesis of the carrier molecule, the amount of lipid-linked oligosaccharides synthesized in cells of the children is reduced, leading to severe hypoglycosylation of proteins. The mutated allels have been expressed in Sec59-deficient yeast in order to demonstrate their loss-of-function.

L058
CDG TYPE I – WHAT CAN WE LEARN FROM MICROBIAL MODEL SYSTEMS?

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N-linked protein glycosylation is affected in the group of Congenital Disorders of Glycosylation (CDG) type I. A common characteristic of these diseases is the absence of glycan chains due to deficiencies in the assembly of the lipid-linked $Glc_3Man_9GlcNAc_2$ oligosaccharide, the substrate for N-linked protein glycosylation by oligosaccharyltransferase in the Endoplasmic Reticulum. The biosynthesis of the lipid-linked oligosaccharide follows a highly conserved pathway in eukaryotic cells and simple microbial model systems, such as the yeast *Saccharomyces cerevisiae*, have been instrumental for the identification of the different enzymes involved in this biosynthesis. This information, in combination with the very similar molecular phenotypes observed in mutant yeast cells and in patient-derived fibroblasts made it possible to identify the molecular cause of many different forms of CDG type I. Studies in yeast revealed why a deficiency in lipid-linked oligosaccharide biosynthesis result in a hypoglycosylation of proteins. At the same time, analysis of the yeast and the recently discovered bacterial N-linked protein glycosylation pathway can lead the way to potential therapeutic concepts aiming at the improvement of N-linked protein glycosylation efficiency in CDG type I cells.

L059
MOUSE MODELS FOR CONGENITAL DISORDERS OF GLYCOSYLATION IA AND IIC

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The most common form of a rapidly expanding group of inherited human disorders which affect the biosynthesis of glycoproteins is Congenital Disorder of Glycosylation Ia (CDG-Ia). More than 500 patients worldwide suffer from this disease which presents with multiple organ defects including severe neurological impairment. CDG-Ia is caused by mutations in the gene encoding for phosphomannomutase 2 (*PMM2*), thereby leading to a reduced conversion of Mannose-6-phosphate to Mannose-1-phosphate. To investigate the pathophysiology of CDG-Ia and to study therapeutic strategies, we first knocked-out the *PMM2* gene in mice and found that the complete loss of PMM2 activity leads to early embryonic lethality. Next, we generated a mouse which expresses the most frequent mutations found in CDG-Ia-patients. Due to a weak residual PMM2 activity these mice are viable and show defects in glycoprotein biosynthesis which are comparable to CDG-Ia patients.
The clinical phenotype of CDG-IIc patients, which is provoked by hypofucosylation of glycoproteins due to a defect in the Golgi GDP-fucose transporter, is mainly characterized by recurrent severe infections with persistent leukocytosis, dysmorphisms, growth and mental retardation. To extend the insight in the general role of protein fucosylation in mammals as well as the pathogenesis of CDG-IIc, we generated a mouse model with a total knock out of the Golgi GDP-fucose transporter. Initial characterization of the mice showed a high postnatal mortality and a severe reduction of GDP-fucose import into microsomal vesicles and of protein fucosylation. Investigation of leukocyte rolling showed a decrease in selectin binding comparable to CDG-IIc patients.

L060
NUCLEOTIDE SUGAR TRANSPORTERS IN CONGENITAL DISORDERS OF GLYCOSYLATION (CDG). A NOVEL HUMAN DISEASE DUE TO INACTIVATION OF THE GOLGI CMP-SIALIC-ACID TRANSPORTER

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The patient had macrothrombocytopenia, neutropenia and lack of the sialyl-Le^x antigen on PMN, he presented spontaneous massive bleedings, due to severe thrombocytopenia (2-6 x 10^9/L), respiratory distress syndrome and opportunistic infections. Transfusions and steroids ameliorated his condition and bone marrow transplantation was performed at 34 months. However, graft-versus-host disease, pulmonary viral infection and massive pulmonary hemorrhage led to death at 37 months. Ultrastructural studies showed hypogranular giant platelets with megakaryocytic hyperplasia.
The Lec2 cells (CHO) are deficient in Golgi CMP-sialic-acid transport due to a splice mutation (IVS6+1G>A). No complementation of these Lec2 cells was obtained, with either of the two patient alleles, whereas full restoration of syalylated phenotype was obtained with the human wild type transcript of the CMP-sialic-acid transporter gene. Inactivation of one patient allele by a double microdeletion inducing a premature stop codon (327) and a splice mutation in the other allele, inducing a 130 bp deletion and a premature stop codon (684), are the causal defects of this disease. An homozygous four base insertion (CACT) in intron 6 was found to be responsible for the splice mutation inducing the 130bp deletion. This CACT insertion, creates a new U2 snRNA site, which is in competition with the normal U2 snNRA site. The addition of this maternal leaky splice mutation to the other completely inactivated allele of paternal origin, induced the disease in the patient. This defect is a new type of congenital disorder of glycosylation (CDG type IIf), affecting the transport of CMP-sialic acid into the Golgi apparatus.

L061
LINK BETWEEN CONGENITAL DISORDERS OF GLYCOSYLATION AND VESICULAR INTRA-GOLGI TRAFFICKING: THE CONSERVED OLIGOMERIC GOLGI (COG) COMPLEX

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Congenital Disorders of Glycosylation (CDG) are a group of rare, genetic disorders characterized by defects in one of the many enzymes, transporters or other functional proteins, required in the glycosylation pathway. Up to now, more than 15 defects have been described, mainly in the endoplasmic

reticulum (Type I) and in the Golgi compartment (Type II). Whereas these CDG cases have defects in genes encoding proteins directly involved in the glycosylation process, recent work has shown that the abnormalities may also be due to abnormal intracellular trafficking of resident Golgi enzymes or transporters involved in glycosylation. Indeed, the function of the Golgi being dependent of the correct localization of all glycosylation enzymes and other resident proteins. This process is achieved by a careful balance between of forward and retrograde traffic of cargo and resident proteins. The Conserved Oligomeric Golgi (COG) complex which is a heterooligomeric tethering complex plays a key role in the retrograde vesicular transport. Two CDG-II patients with two different COG deficiencies (COG1 and COG8) were found. They have a multi-system disease with severe neurological involvement. Both of them have a combined N- and O-glycosylation defect characterized mainly by hyposialylation. The mutations destabilize other COG subunits, and affect the subcellular localization and hence the overall integrity of the COG complex. It further suggest that the interaction between COG1 and COG8 play a central role in the formation and the functionality of the whole COG complex. Deficiencies in this complex thus define a new group of glycosylation disorder.

L062
CONGENITAL MUSCULAR DYSTROPHIES DUE TO GLYCOSYLATION DEFECT OF PROTEIN *O*-MANNOSYLATION

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Muscular dystrophy is a group of inherited myogenetic disorders characterized by progressive muscle weakness and wasting. Muscle-eye-brain disease (MEB), Walker-Warburg syndrome (WWS), and Fukuyama congenital muscular dystrophy (FCMD) are clinically similar autosomal recessive disorders characterized by muscular dystrophy, lissencephaly, and eye anomalies. The expression of the highly glycosylated α-dystroglycan is abnormal in all forms, implicating that defective glycosylation of this protein is central to the pathogenesis of these disorders. *O*-Mannosylation is rare protein modification and one of the best-known *O*-mannosyl-modified glycoproteins is α-dystroglycan. The *POMGnT1* gene is responsible for MEB and the *POMT1* and *POMT2* genes are for WWS. POMGnT1 is responsible for the formation of GlcNAcβ1-2Man. POMT1 and POMT2 are both required for protein *O*-mannosyltransferase activity. These findings suggest that defective protein *O*-mannosylation of α-dystroglycan is associated with MEB and WWS. FCMD is thought to result from a similar aberrant glycosylation of α-dystroglycan. As abnormal glycosylation of α-dystroglycan is also found in a number of yet uncharacterized conditions, the list of glycosyltransferases responsible for muscular dystrophies will considerably increase over the following years.

L063
BINDING PROPERTIES AND STRUCTURAL ANALYSIS OF CD33-RELATED SIGLECS EXPRESSED IN THE INNATE IMMUNE SYSTEM

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The CD33-related siglecs represent a rapidly evolving subfamily of the siglec group of sialic acid binding Ig-like lectins whose repertoire differs significantly between mammalian species. In humans there are up to 8 *bona fide* members whereas in mice there are only 5. The CD33-related siglecs are expressed mostly on different cells of the innate immune system, where they interact with sialylated glycans *in cis* and have the potential to modulate leukocyte activation and apoptosis. In order to gain insights into structure-function relationships of the CD33-related siglecs in mice and humans, they were expressed as recombinant Fc-chimeras and provided to the Glycan Array of the Consortium for Functional Glycomics. The results reveal striking differences for each CD33-related siglec, with overlapping specificities shared between putative orthologues of mice and humans that give insights into the complex evolutionary relationships between these proteins. The specific binding properties have been further analysed in secondary binding analyses, both to defined glycoconjugates and to cells of the immune system. Finally, we have begun to explore the molecular basis for glycan recognition by CD33-related siglecs using X-ray crystallographic approaches with the natural killer cell siglec, siglec-7 as a model system. Our results reveal how complex oligosaccharides can be specifically bound by CD33-related siglecs and suggest that a remarkable degree of plasticity can exist for carbohydrate recognition by this family of proteins.

L064
IMPACT OF THE STRUCTURAL FEATURES OF LEWIS CARBOHYDRATES ON ANTIBODY TARGETING OF CANCER

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Introduction. Lewis carbohydrate antigens are expressed at high levels in many carcinomas, including those of colon, breast, lung, prostate and ovary. In particular, type 2 (Le^x and Le^y) antigens are targets for cancer immunotherapy. We recently determined the 3D structure for the interaction between a humanized antibody (hu3S193) and its Le^y tumor antigen [Ramsland et al. (2004) *J. Mol. Biol.* 340:809-18]. Here we review the conformational nature of free and bound Lewis oligosaccharides and their interactions with antibodies. *Experimental procedures.* Dihedral angles of the glycosidic linkages were compared for available structures of Lewis oligosaccharides. Detailed structural comparisons were performed using the crystallographic coordinates of antibodies (hu3S193, BR96 and 291-2G3-A) in complex with type 2 Lewis antigens. *Results.* Structural studies consistently show that free Lewis oligosaccharides are rigid structures with very similar shapes. Their conformations are generally maintained in the bound state. The hu3S193 and BR96 antibodies bind Le^y carbohydrate epitopes in almost identical ways with some subtle differences. In contrast, the 291-2G3-A antibody binds its Le^x ligand in a comprehensively different manner. However, all three antibodies are specific for their respective type 2 ligands and furnish multiple interactions with key functional groups of the core GlcNAc residue. *Conclusion.* A structural basis for antibody targeting of type 2 Lewis antigens is emerging and appears to be driven (at least for Le^y specific antibodies) by a structurally convergent immune response. The structural information is of potential use in the ongoing development of carbohydrate specific antibodies for immunotherapy of cancer.

L065
INTERACTIONS OF A POLYSIALIC ACID 'ANTIBODY MIMIC' CONSTRUCTED FROM INACTIVE ENDOSIALIDASE

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Polysialic acid is a neurodevelopmental antigen of the neural cell adhesion molecule NCAM, as well as a capsular polysaccharide of meningitis-associated bacterial pathogens. It is cleaved by endosialidases derived from *E. coli* bacteriophages. Bacteriophage mutants isolated by natural selection encode endosialidases which have a reduced or lost enzymic activity, but are still capable of binding with high affinity to their substrate, polysialic acid. Double or triple amino acids substitutions in the endosialidase mutants were identified by sequencing of their cloned genes. The contributions of individual amino acids were evaluated by expressing constructs with single amino acid substitutions. In a homology-based structural model the critical amino acid residues map close to each other in the catalytic domain of the enzyme, which suggests their involvement in the endosialidase activity of the enzyme. A mutant inactive endosialidase fused to green fluorescent protein via a spacer is an efficient and specific 'antibody mimic', which has been useful in the detection of polysialic acid during brain development, in neuroblast tumours, in bone repair, and in pathogenic bacteria.

Jokilammi, A.; Ollikka, P.; Korja, M.; Jakobsson, E.; Loimaranta, V.; Haataja, S.; Hirvonen, H.; Finne, J. (2004): Construction of antibody mimics from a noncatalytic enzyme - detection of polysialic acid.
J. Immunol. Methods 295, 149-160.

L066
MHC-GUIDED PROCESSING OF A DIFFERENTIALLY FOLDED GLYCOPROTEIN TO INDUCE ANTITUMOR CD4 T CELL RESPONSE.

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Glycoprotein folding in the endoplasmic reticulum (ER) is subject to a quality control system involving the lectin-like chaperones calreticulin (CRT) and calnexin. Only native conformers reach their final destinations, and non-native conformers and incompletely folded proteins are retained in the ER. However, the immunological consequences of the generation of incompletely folded conformers *in vivo* have not been fully understood. Recently, we have found an MHC class II-restricted tumor antigen whose antigenicity is conformation-dependent and correlates with CRT binding [1]. Endogenous MuLV envelope glycoprotein gp90 synthesized by colon carcinoma CT26 cells stimulates a panel of CD4 T cell hybridomas generated by immunization of autologous BALB/c mice with CT26 [1] but gp90 from other BALB/c mouse-derived tumors is not stimulatory although their gp90 DNA sequence is identical. We investigated the molecular basis for differential presentation of gp90 by MHC class II molecules. We detected a unique folding status of the CRT-bound gp90 from CT26 by nonreducing SDS-PAGE and the presence of monoglucosylated glycans in antigenic gp90 by HPLC analysis. The CRT-bound form was found to be captured by mature, recycling $I\text{-}A^d$ MHC class II molecules on the surface of antigen presenting cells in the “bind then trim” model in contrast to the “trim then bind” model in which a protein antigen is enzymatically cleaved to short peptides before MHC loading. This result indicates that the generation of an immune response may in some instances be controlled by the intracellular folding environment and MHC-guided processing.

Reference

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L067
THE GLYCOIMMUNOLOGY OF GUILLAIN BARRÉ SYNDROMES

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Autoimmune neuropathies are post-infectious paralytic syndromes caused by inflammation in peripheral nerves. Guillain Barré syndrome (GBS) and the variant Miller Fisher syndrome (MFS) are the prototypic syndromes and the foremost cause of neuromuscular paralysis worldwide. Recent clinical-serological studies have shown that serum anti-GM1/GD1a antibodies characterise the motor axonal form of GBS whereas anti-GQ1b/GT1a antibodies characterise the Miller Fisher syndrome (MFS). Antibodies may be induced by immune responses to cross-reactive epitopes borne by infectious organisms, including *Campylobacter jejuni* lipooligosaccharides. In these disorders anti-ganglioside antibodies target gangliosides in neuronal and glial membranes, including nodes of Ranvier and motor nerve terminals. Using *in vivo* and *ex vivo* mouse neuromuscular junction preparations as a model system, we have elucidated one of the mechanisms by which antibodies cause neural injury. At nerve terminals, dense antibody and complement deposits are found in the synaptic cleft and on perisynaptic Schwann cells (pSCs), alonsgside destruction of the nerve axons, and pSC death. Neural injury can be exaggerated by increasing the local concentration of ganglioside, and in complement regulator deficient mice. Complement inhibition can attenuate the pathological procession. GM2/GD2 synthase null-mutant mice that lack complex gangliosides are unaffected, thereby demonstrating that complex gangliosides are the neuronal targets for anti-ganglioside antibodies in this system. Therapeutic modulation can also potentially be achieved by blocking anti-ganglioside antibodies with synthetic oligosaccharides, either in solution or as part of a selective immunoadsorption therapy.

L068
CARBOHYDRATE MIMICRY IS A CAUSE OF AUTOIMMUNE DISEASES

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Molecular mimicry between microbial and self components is postulated as the mechanism that accounts for the antigen and tissue specificity of immune responses in post-infectious autoimmune diseases. Little direct evidence exists, and research in this area has focused principally on T cell-mediated, anti-peptide responses, rather than on humoral responses to carbohydrate structures. Guillain–Barré syndrome (GBS) is the prototype of postinfectious autoimmune diseases. Gram-negative bacterium *Campylobacter jejuni* is the most frequent agent of antecedent infection in GBS, and autoantibody to GM1 or GD1a is associated with GBS subsequent to *C. jejuni* enteritis. Molecular mimicry has been found between human gangliosides GM1/GD1a and the lipo-oligosaccharide (LOS) of *C. jejuni* isolated from a GBS patient. Sensitization of rabbits with *C. jejuni* LOS, as well as GM1, induced production of anti-GM1 IgG antibody and subsequent development of acute flaccid paralysis. Pathological changes seen in rabbit peripheral nerves were identical to those seen in GBS. Knockout mutants of *C. jejuni* genes involved in LOS sialylation reduced reactivity with anti-GM1 sera from GBS patients, and did not induce an anti-GD1a IgG antibody response in mice. LOS biosynthesis genes appear to be essential for the induction of anti-GM1 or anti-GD1a IgG antibody and the subsequent development of GBS. These findings provide conclusive evidence that molecular mimicry is a cause of human autoimmune disease. This new concept that carbohydrate mimicry can cause an autoimmune disease provides a clue to the resolution of the pathogenesis of other immune-mediated diseases.

L069
SYNTHETIC SULFATED GLUCURONOSYLPARAGLOBOSIDE (SGPG) AND ITS USE FOR THE DETECTION OF AUTO-IMMUNE PERIPHERAL NEUROPATHIES.

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The sulfated glucuronosylparagloboside .SO4-3-GlcA(β1−3)Gal-(β1−4) GlcNAc(β1−3)Gal-(β1−4)Glc(β1-1')Cer (SGPG) is a specific constituent of the peripheral nervous system. The terminal trisaccharide sequence of the SGPG (N-acetylglucosaminyl – galactosyl – glucuronylsulfate is the HNK-1 epitope, common to the SGPG, and its derivatives and also to several adhesion molecules and myelin proteins among which MAG (myelin-associated glycoprotein). Predominantly sensory demyelinating neuropathies with monoclonal immunoglobulins of the M type (IgM) have antiMAG/SGPG activities, whereas axonal neuropathies, predominantly motor, or some cases of motor neuron diseases with monoclonal IgMs, present only anti-

SGPG activity. Thus there is a broad spectrum of dysimmune neuropathies characterized by antiSGPG antibodies. SGPG is not commercially available and, as it is only a minor constituent of PNS of several animal species, its purification remains a hard task. For this reason, it appears important for clinical purposes to synthesize this glycolipid. This has been performed using a new chemical synthesis with a stearic tail for the ceramide moiety. The synthetic compound was characterized by mass spectrometry. The synthetic product, although containing a single fatty acid, has the same specificity for IgM SGPG antibodies than the natural product. The ELISA test set up for the detection of SGPG antibodies proved to be more sensitive than the search for anti-MAG antibodies. and allowed the detection of dysimmune demyelinating neuropathies, in the past, undiagnosed. This may have therapeutic consequences as treatments are being more and more specific and designed to lower antibody concentration using synthetic and specific oligosaccharide epitopes or to interfere with immunopathogenic mechanisms.

L070
GLYCOSYLATION OF HUMAN C-REACTIVE PROTEINS (CRP) MODULATES THEIR BIOLOGICAL ROLE

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Introduction: Human CRP, an acute phase protein, is massively induced as a part of the innate immunity to inflammation. However, the role of CRP in establishment of infection or disease remains unexplored. Previous studies convincingly demonstrated that human CRPs are induced in a disease-specific manner and differentially glycosylated in certain pathological conditions. Investigations pertaining to the direct association of glycosylated molecular variants of CRP to *Leishmania donovani* parasite and diseased erythrocytes deserve attention in the era of CRP-glycobiology in view of host-parasite interaction for parasite invasion and the clearance of erythrocytes.
Methodolgy: The binding of parasites and erythrocytes from malaria, tuberculosis and leishmaniasis with purified CRPs from respective patient's sera was confirmed through Flow-cytometric analysis, radiobinding assays, ELISA and Western blot analysis. The apparent binding constants and the number of receptors were calculated from Scatchard plot. Additionally, a new molecule antigenically-related to CRP was detected on the surface of parasite.
Results: CRPs bind 2-3 folds stronger with diseased-erythrocytes than normal individuals in a disease-specific manner. Deglycosylated CRPs showed altered binding. Alteration in the fragility, rigidity and hydrophobicity of diseased-erythrocytes was observed after CRP binding which triggers the CRP-complement pathway causing hemolysis. Additionally, role of CRP in the binding of the parasites and their uptake by macrophages was also observed.
Conclusions: This study provides a new dimension to the functional-interaction of CRP as a contributory factor of anemia in these three diseases. A new potential role of CRPs and newly identified CRP-like molecules has been established in invading the host cells in a disease-specific manner.

L071
ROLE OF SIALOADHESIN IN IMMUNE REGULATION

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Sialoadhesin (Sn) is a highly conserved cell surface molecule that belongs to the Siglec (Sialic acid binding Ig-like lectin) family. Expression of Sn is restricted to subsets of tissue macrophages, particularly in secondary lymphoid tissues, and inflammatory macrophages. It has previously been shown to bind a variety of cells *in vitro*. In order to address its function *in vivo* we created Sn-deficient mice. Sn was completely absent from spleen and lymph node sections and was undetectable by FACS analysis, confirming that these mice were true nulls.
Comparison of cell populations in spleen, peritoneal cavity, bone marrow, lymph node and blood of Sn-deficient and wild type mice by flow cytometry showed only subtle alterations in T and B cell subsets in spleen and lymph nodes with no significant differences in myeloid cell compartments. We further screened for antibody isotypes in unstimulated mice and found decreased IgM levels in Sn-deficient mice while no major deviations for IgG subtypes were observed. The IgM response was also diminished after immunising with T-dependent or T-independent antigens. Surprisingly, in T-dependent antibody responses this did not affect IgG production. As expected, we did not find any differences in antibody production or proliferation after B cell stimulation *in vitro*.
From the present data we can conclude that Sn has a role in selectively supporting IgM production. This phenotype is very interesting and might also give new insight into the regulation of humoral immune response.

L072
IMINOSUGARS AS ANTIVIRALS FOR HEPATITIS B & C

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We are employing two different strategies to develop antiviral therapies using iminosugar derivatives. The first approach involves targeting virus encoded protein(s), the second targets host cell encoded protein(s) necessary for virus survival. The latter could potentially prove more resistant against the development of viral escape mutants, a problem plaguing most conventional drug therapies.
In the case of hepatitis C virus (HCV), which affects about 3% of the world population, both strategies are used. Using bovine viral diarrhoea virus (BVDV) as a model organism for HCV we showed that inhibition of the host cell ER alpha-glucosidases I and II using the glucose analogue NB-butyl deoxynojirimycin (NB-DNJ) leads to an antiviral activity caused by a reduction in viral secretion due to the interference with viral envelope glycoprotein folding and subsequent impairment of viral morphogenesis. However, it is also possible to target the virally encoded HCV protein p7 required for viral assembly and secretion. P7 can form ion channels which can be inhibited using long alkylchain imino sugar derivatives.
Worldwide, more than 350 million people are chronically infected with hepatitis B virus (HBV). Glucosidase inhibitors are antiviral against HBV in tissue culture and in the woodchuck model of chronic HBV infection. The M surface antigen glycoprotein of HBV folds via the calnexin pathway. Glucosidase inhibitors prevent this interaction and prevent the formation and secretion of HBV. The misfolded M surface antigen is retained within the cell and may itself act as a long lived "drug" which prevents virus formation.

L073
CARBOHYDRATE RECOGNITION AS A TEMPLATE FOR HIV VACCINE DESIGN

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A small number of antibodies from HIV-infected individuals provide broad sterilising immunity against viral challenge in animal models. We have previously characterised the epitope of 2G12, a neutralizing anti-HIV IgG1 that binds to an unusual cluster of Manα1-2Man residues within the *N*-linked glycans of HIV-1 gp120 (Ki = 0.5 mM). An immunogen capable of eliciting 2G12-like antibodies may be expected to confer significant protection against natural HIV infection. However, the relatively poor immunogenicity of the oligomannose glycans on gp120 is a barrier to the design of a carbohydrate vaccine for HIV-1. We have approached this problem using three different strategies to achieve multivalent presentation of the 2G12 glycan epitope.
CHO cells expressing gp120 were incubated with the ER α-mannosidase I inhibitor kifunensine [5 μM] to create a novel gp120 immunogen with at least two high affinity 2G12 epitopes ($Man_9GlcNAc_2$). Presentation of multiple copies of the 2G12 epitope on the gp120 scaffold may increase the immunogenicity in a subunit vaccination strategy.
The oligomannose glycans ($Man_{5\text{-}9}GlcNAc_2$) of gp120 are not antigenic, however synthetic mannosides containing Manα1,2Man were recognized by IgG, IgA and IgM in serum from healthy controls and infected patients. Multivalent conjugates of these synthetic antigens showed enhanced 2G12 binding (Ki = 0.5 μM).
Yeast mannans are highly immunogenic, but not significantly antigenically cross-reactive with the mannose structures present on HIV-1. However, we have demonstrated that 2G12 binds clustered Manα1-2Man residues on *S. cerevisiae* deficient in α1-3 mannosyl transferase (Mnn1p). In an evolutionary approach using repeated rounds of mutation and selection, we have shown that 2G12 binding is a selectable phenotype.

L074
NOVEL STRATEGIES FOR A MELANOMA VACCINE

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Melanoma is one of the most malignant forms of cancer that is now at epidemic proportions worldwide. This form of cancer begins in the transformed melanocytes of the skin prior to being spread and disseminated at distant metastatic sites. That the immune system might recognize and reject melanoma, has been suggested by the finding of melanoma antigen specific cytotoxic T-lymphocytes (CTLs) in the serum of melanoma patients. However, despite the existing evidence that pre-activated T cells can recognize tumor cells, there is an yet unknown mechanism that reduces their efficacy in melanoma patients. To initiate an antigen-specific immune response, *processing pathways* converting the tumor protein into peptides that bind to MHC molecules are required.
We found that tyrosinase is a melanoma specific antigen whose glycans direct its folding and processing in the endoplasmic reticulum(ER) based on their interaction with the chaperones calnexin and calreticulin. Melanoma tyrosinase transits the ER en route to melanosomes and only some of the

molecules are retained in the ER and degraded. To increase the presentation efficiency we designed tyrosinase mutants that are completely retained in the ER and degraded efficiently. These mutants lack either the transmembrane domain of tyrosinase, or essential N-glycans. Both the TM domain and specific N-glycans were found to facilitate the calnexin interaction of the molecule and hence its folding process. Therefore, the novel strategy that we propose is to create a melanoma vaccine based on mutants designed to be retained in the ER and degraded to get an efficient immune response of CD8+ T cells.

L075
NEW TECHNOLOGIES FOR CANCER DIAGNOSTICS

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Although many cancers can be treated and cured if they are diagnosed while tumors are still localized, most cancers are not detected until after they have invaded the surrounding tissue or metastasized to distant sites. As more therapeutic options for cancer treatment become available there is an increasing need for new biomarkers that will provide more sensitive and specific early detection of cancer. In addition, improved technologies for monitoring disease progression and response to therapy are required. Assays of existing serum biomarkers are neither sensitive nor specific enough for use as the sole screening method. A strategy has been developed to enable cancer-related biomarkers to be identified from detailed analysis of at least 40 N-linked oligosaccharides that comprise the serum glycome of healthy controls and cancer patients (including prostate, pancreatic, ovarian, breast and hepatocellular carcinoma). Cancer associated proteins, such as inflammatory markers, contribute to the overall changes in the serum glycosylation profile. In addition, glycosylation processing pathways are usually disturbed in tumors, therefore tumor related secreted glycoproteins can be identified that also contribute to the differences in glycan profiles. Rapid, high throughput, optimized screening technologies allow the quantitative determination of specific glycan markers in less than 1µl of serum, for example by combining HPLC based glycan profiling of whole serum with enzyme array digestions to segregate the markers. Automated data analysis is fine tuned for each form of cancer. These technologies may prove useful for diagnosing cancer and monitoring disease progression and therapy.

L076
CHEMISTRY AND BIOLOGY OF IMINO SUGARS

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Imino sugars are endocyclic nitrogen mimics of monosaccharides and when modified with N-alkyl chains, have structural similarity to ceramide. As a consequence, imino sugars inhibit both exoglycohydrolase and ceramide glucosyltransferase activities leading to their exploitation as potent therapeutics for viral infections and glycolipid storage disorders. Despite this utility, the precise mechanism of action, entry of imino sugars to cells, cell organelles and tissues, especially the CNS, is poorly understood.
Using *in vitro* and cell based assays we have analysed enzyme inhibition by imino sugars that are used to target different cell organelles. Imino sugar entry to cells was determined by the extent of ceramide glucosyltransferase inhibition; access to the endoplasmic reticulum (ER) lumen evaluated by analysing free oligosaccharides (FOS) produced as a result of ER-glucosidase inhibition, and lysosomal uptake analysed by glycolipid and FOS accumulation. Modifications to alkyl chain length were used to probe the effects on the CNS in additional studies *in vivo* and confirm that the restricted ability of imino sugars to cross the blood/brain barrier can be improved by increasing hydrophobicity.
These data reveal the rates of access of imino sugars to intracellular compartments and have profound implications for targeting imino sugars to enzymes. Optimal treatment of glycolipid lysosomal storage diseases using imino sugars for substrate reduction therapy (SRT) or chaperon-mediated therapy (CMT), and the use of imino sugars as anti-viral agents for HIV and hepatitis, requires further development and improvement to enzyme selectivity, cellular localisation and tissue uptake.

L077
POLYSIALIC ACID IN FORMATION AND REPAIR OF THE NERVOUS SYSTEM

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Polysialic acid (PSA) expression on the neural cell adhesion molecule (NCAM) is known to attenuate contact-dependent cell-cell interactions. Surface-force measurements between lipid bilayers containing PSA-NCAM establish that this reduction in interactions directly reflects precisely controlled repulsive forces between the two apposing surfaces.
The presence of PSA-NCAM on cells in the vertebrate embryo has been found to promote tissue plasticity with respect to precursor cell migration, and the growth and guidance of axons. Because most regions of the adult CNS no

longer express PSA, they lack this type of plasticity. Accordingly, we have conducted studies to determine if viral delivery of PSA-synthetic capability into regions of nerve damage can facilitate the restoration of connections. Studies using this delivery system has revealed that ectopic PSA can disrupt glial cell interactions, which suggested that it might help to reduce the impact of glial scars known to inhibit the passage of axons. Subsequent work has demonstrated that such a mechanism can in fact promote growth of lesioned axons through the scar with significant efficiency. Similarly, we have found that experimental induction of PSA in a pathway leading from the subventricular zone, which contains precursors even in the adult animal, to a lesion site in the cortex, results in migration of these cells into the region of damage.

L078
THE PHENOTYPE OF POLYSIALIC ACID NEGATIVE MICE

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Polysialic acid (polySia) is a dynamically regulated posttranslational modification of the neural cell adhesion molecule (NCAM) and essentially implicated in neural development, regeneration, and plasticity. Two independently regulated polysialyltransferases ST8Sia- II and ST8Sia-IV are the key enzymes in polySia biosynthesis.
With the aim to separately investigate polySia and NCAM functions, mice genetically depleted in both polysialyltransferases were generated by crosses between the single knockouts *St8siaII* and *St8SiaIV*.
Mice lacking both polysialyltransferases were born apparently normal but failed to thrive and precociously died. Western blot analysis of brain samples showed that polySia-biosynthesis was completely abolished, while NCAM expression seemed to be unchanged. Unexpectedly polysialyltransferase-depleted mice share phenotypic alterations (e.g. small olfactory bulbs in conjunction with disturbed neuroblast migration in the rostral migratory stream and delamination of the hippocampal mossy fiber tract) with NCAM-knockout mice, which lack both polySia and NCAM. However, beyond the NCAM-knockout phenotype polysialyltransferase-negative mice show early lethality, progressive hydrocephalus and severe affections of commissural and non-commissural fiber tracts. The lethal phenotype observed could be rescued after additional deletion of the NCAM-gene. In summary, the data obtained in this study clearly demonstrate that polySia has an essential function in controlling NCAM interactions during mouse development.

L079
SIALIC ACID PRECURSORS ARE TOOLS TO INTERFERE WITH POLYSIALYLATION OF THE NEURAL CELL ADHESION MOLECULE IN VITRO AND IN VIVO

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Sialic acid (Sia) is expressed as terminal sugar in many glycoconjugates and plays an important role during development and regeneration. Addition of homopolymers of Sia (polysialic acid = polySia) is a unique and highly regulated posttranslational modification of the neural cell adhesion molecule (NCAM). The presence of polySia affects NCAM-dependent cell adhesion and plays an important role during brain development, neural regeneration and plastic processes including learning and memory. Polysialylated NCAM is expressed on several neuroendocrine tumors of high malignancy and correlates with poor prognosis. Two closely related enzymes, the polysialyltransferase ST8SiaII and ST8SiaIV, catalyze the biosynthesis of polySia. This presentation summarizes recent approaches to modify the degree of polySia on NCAM in vitro and in vivo. First, we describe the selective inhibition of ST8SiaII using synthetic sialic acid precursors. Second, we demonstrate that the key enzyme of the sialic acid biosynthesis (UDP-N-acetylglucosamine 2-epimerase = GNE) regulates and limits the synthesis of polysialic acid.

L080
DEVELOPMENT OF THERAPEUTIC APPLICATIONS FOR *N*-ACETYLMANNOSAMINE (MANNAC) ANALOGUES USED FOR THE MANIPULATION OF SIALIC ACID METABOLISM.

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The biochemical engineering of sialic acid, through the use of non-natural analogues of *N*-acetylmannosamine (ManNAc)

that manipulate sialic acid metabolism, has potential applications in tissue engineering, regenerative medicine, infectious disease, and cancer therapy. The effects of ManNAc analogues on cells are widespread and include both intracellular and cell surface changes. Inside a cell, the analogues perturb the levels of sialic acid pathway intermediates, they modify gene expression, and they modulate apoptosis. Upon metabolic conversion of the analogue to the corresponding sialic acid and its subsequent incorporation into cell surface-displayed sialoglycans, a cell can be given novel physical and chemical properties that – depending on the exact *N*-acyl modification of the analogue under test – can be exploited to achieve novel modes of cell adhesion of benefit to tissue engineering, to prevent viral binding, to install novel epitopes that change the surface immunochemistry, and to serve as 'chemical handles' for the delivery of diagnostic or therapeutic agents. A difficulty in translating this methodology to clinical practice is that ManNAc analogues, like sugars in general, are not 'drug-like.' A common strategy to improve the pharmacological properties of carbohydrates is the per-acetylation of their hydroxyl groups; our laboratory has shown that derivation of hydroxyl groups with protecting groups other than acetate can not only further improve the efficiency of analogue utilization by a cell but can also provide the sugar with novel biological properties. As a consequence, the repertoire of cellular behaviors that can by manipulated by sialic acid engineering methods has been considerably increased.

L081
SIALIC ACID RECOGNITION IN AXON STABILITY AND AXON REGENERATION

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Complementary interacting molecules on neuronal axons and the myelin membranes that ensheathe them are required for long-term axon stability. Disruption of axon-myelin molecular interactions results in axon degeneration, contributing to the pathogenesis of demyelinating diseases, such as multiple sclerosis. Myelin-associated glycoprotein (MAG), a minor constituent on the periaxonal surface of central and peripheral nervous system myelin, is a member of the Siglec family of sialic acid-binding lectins. MAG, on myelin, binds to two major sialylated glycosphingolipids expressed on the axon surface, gangliosides GD1a and GT1b. Mice lacking the ganglioside biosynthetic gene *Galgt1* fail to express complex gangliosides, including GD1a and GT1b. We found that *Mag*- and *Galgt1*-null mice exhibit similar central and peripheral nervous system axon degeneration, decreased neurofilament spacing, and decreased axon diameter. *Mag/Galgt1* double-null mice displayed similar pathology. Consistent with these findings, *Mag*- and *Galgt1*-null mice displayed similar motor and behavioral deficits. These data demonstrate that MAG and complementary gangliosides contribute to axon stability in the central and peripheral nervous systems. In addition to stabilizing axons, MAG, on residual myelin membranes, blocks axon regeneration at sites of nervous system injury. MAG-mediated inhibition of axon regeneration is dependent on sialoglycoconjugates. Pre-treatment of neurons with sialidase reverses MAG-mediated inhibition in vitro, as does addition of disialyl T-antigen, a high-affinity MAG-binding glycan. Taken together, these data demonstrate that sialoglycans are functional ligands for MAG, and are involved both in axon-myelin stabilization and inhibition of axon regeneration after nervous system injury. Supp. by National Institutes of Health, Bethesda, MD USA.

L082
DYNAMIC CHANGE IN SIALIC ACID SPECIES IN MOUSE GERMINAL CENTER B CELLS

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Sialic acid is involved in the maintenance of the negative charge of cellular membrane or glycoconjugates, and also in the molecular recognition events. In mice, sialic acid occurs mainly in two forms, *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). Neu5Gc is biosynthesized from Neu5Ac by hydroxylation of the *N*-acetyl group at the C-5 position. CMP-Neu5Ac hydroxylase, which converts CMP-Neu5Ac to CMP-Neu5Gc, is responsible for the synthesis. Neu5Gc is involved in the CD22 (Siglec-2)-mediated recognition in mouse immune system, because CD22 requires Neu5Gc for its ligand. Mice with disrupted CMP-Neu5Ac hydroxylase gene (*CMAH*) were characterized. B cells from the mice showed hyper-responsive phenotypes, suggesting that the recognition of the properly sialylated ligand(s) by CD22 functions as a negative modulator of B cell receptor signaling. For further analysis of these *CMAH*-deficient mice, we examined flow cytometric profile of B cells using activation-dependent markers. Among them, GL7, a marker for germinal center, is strikingly positive in *CMAH*-deficient B cells. Moreover, in immunized wild-type mice, germinal center B cells suppressed CMP-Neu5Ac hydroxylase, resulting in the loss of Neu5Gc, that is, CD22-ligand. From these results, we concluded that germinal center B cells escape from the negative signaling through CD22 binding to Neu5Gc-bearing ligand(s) by down-regulation of Neu5Gc.

L083

NOVEL CLUES IN THE PATHOGENESIS OF HEREDITARY INCLUSION BODY MYOPATHY

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Hereditary Inclusion Body Myopathy (HIBM), an autosomal recessive muscular disorder, is caused by mutations in the gene encoding the bifunctional enzyme UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE), the key enzyme in the biosynthetic pathway of sialic acid. Several missense mutations were identified in *GNE* in HIBM patients worldwide, whereas a unique homozygous founder mutation, M712T, was found in all Middle Eastern patients. Despite intensive cellular and biochemical characterization of HIBM muscle cells, the pathophysiological pathway leading from GNE mutations to HIBM is poorly understood. In order to elucidate the first occurring downstream events of this pathway we have analyzed the gene expression patterns of muscle specimens from 10 HIBM patients, carrying the M712T mutation, presenting mild histological changes, and 10 healthy individuals. 374 genes were identified as significantly differentially expressed between affected and healthy individuals. Among those, a large number of transcripts encoding proteins (18%) are involved in the oxidative phosphorylation cascade, ATP synthesis, apoptosis and other mitochondrial processes. These data point to the possible involvement of the mitochondrial apoptosis pathway, known to be often initiated by gangliosides effectors, in the pathophysiology of HIBM. To date GNE has been related solely to the sialic acid biosynthetic pathway, and no clear understanding of the cause/effect relationship between GNE mutations and HIBM phenotype could be provided. The identification of a mitochondrial expression signature in HIBM affected muscles, if confirmed, could disclose new functions for GNE in muscle cells.

L084
BIOACTIVE GLYCAN-ARRAYS IN GLYCOMICS

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Under the auspicious of the Consortium for Functional Glycomics (CFG) we have recently developed a novel glycan array format that employs covalent coupling of glycans to glass slides. The array utilizes standard robotic printing technology and commercial amine reactive N-hydroxy-succinimido (NHS) activated glass slides which allows rapid covalent coupling of more than 250 diverse amine functionalized synthetic and natural glycans or glycoconjugates[1].

In the present work, we demonstrate the utility of the printed glycan array from various collaborative projects such as: (A) The anti-carbohydrate antibody response in patients transplanted with xenogeneic fetal pig islet-like cell clusters. Between 1990 and 1993, 10 patients with type I diabetes were transplanted with porcine fetal islet-like cell clusters (ICC) (2). Following transplantation of fetal pig islets, there was a general titre rise among the various anti-carbohydrate Ab specificities detected. Some Ab specificities, for example against certain α-Gal- and Neu5Gc-structures, appeared as a result of transplantation. (B) Evaluating glycan reactivities of Group B streptococci (GBS) monoclonal antibodies. The type-specific capsular polysaccharide (CPS) antigen is the major protective antigen for GBS. To understand the complexity of the immune response to this antigen, we have analyzed a panel of mAbs to type III CPS with glycan array. Each antibody produced a unique pattern of binding. GD3/GT3 gangliosides, found on cell surfaces and developing neural tissue, were identified as binding to protective anti-CPS_{III} mAbs. Epitopes bound by protective antibodies contain sialic acid. Other antibodies bind entirely different carbohydrate structures.

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L085
DESIGNER MICROARRAYS FOR STRUCTURAL AND FUNCTIONAL GLYCOMICS

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The diverse oligosaccharides that 'decorate' glycoproteins, glycolipids, proteoglycans and polysaccharides are potentially a vast source of information, and could harbour 'glyco codes' that are waiting to be deciphered in various contexts of biological and medical importance. It is desirable therefore to develop a knowledge-base of biological systems that operate through oligosaccharide recognition. Toward this goal a carbohydrate microarray technology [1] has been introduced

that includes oligosaccharide probes with lipid tags (neoglycolipids) [2]. These can be generated from natural and synthetic sources. Thus, the carbohydrate microarrays can be designed from diverse origins, e.g. bacteria, cells and organs that are targeted by particular carbohydrate-binding proteins. Coupled with mass spectrometry, this approach lends itself to surveys of entire glycomes and proteomes for the molecular definition of carbohydrate-recognition systems in whole organisms.

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L086
GLYCAN MICROARRAYS ON THE BASIS OF THREE-DIMENSIONAL HYDROGELS

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Three-dimensional hydrogel-based microchips with immobilized DNA and proteins were developed in the Engelhardt Institute of Molecular Biology, Moscow, Russia. Microchips consist of hydrogel nanoliter drops separated with hydrophobic surface. Covalent binding of probes containing active groups (amino, sulfhydryl, etc) with the components of growing polymer chain occurs during polymer formation.

Hydrogel-based glycan microarrays were manufactured. Spacered amino-saccharides and polyacrylamide glycoconjugates (PAA-Sug) were taken for immobilization. Procedures for carrying out assays on glycan microchips involving mixing of the reaction solution were developed. Mixing resulted in increasing the sensitivity of the assay and shortening the reaction time.

Glycan microchips were used for quantitative immunoassay of antibodies against blood group trisaccharides in cultural fluid and in blood serum and for studies of glycan specificity of lectins. In all cases, only specific interactions with chip-immobilized saccharides were observed. The specificity of binding of ricin, ricin toxic subunit, and ricin agglutinin with carbohydrate ligands was studied using combined glycan and antibody microchips. The association constants for binding of ricin with chip-immobilized ligands were determined.

The developed in EIMB universal technology of hydrogel microchips manufacturing is suitable for the preparation of DNA, protein, glycan, and combined microchips. Saccharides immobilized in hydrogel drops may be considered as an analog of glycoconjugates with hydrogel polymer instead of PAA that are attached to the glass surface. Hydrogel glycan microchips can be used for carrying out different types of assays and studies of glycan specificity of proteins.

L087
CARBOHYDRATE CHIPS FOR FUNCTIONAL GLYCOMICS

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Cell-surface carbohydrates are involved in a variety of important biological processes, such as cell adhesion, fertilization, differentiation, development and tumor cell metastasis through carbohydrate-protein interactions. Interestingly, it is through these biomolecular interactions that bacteria and viruses adhere to the host cells and confer pathogenic properties. Therefore, it is important to determine the molecular basis for specific protein-carbohydrate recognition events. Details of carbohydrate-protein interactions have been investigated mainly by biophysical and biochemical approaches. Despite the established approaches to investigate carbohydrate-protein interactions, high-throughput methods have not been developed. As attempts to develop novel tools for studying high-throughput carbohydrate-protein interactions, we have fabricated various types of carbohydrate chips by immobilizing carbohydrates on the properly derivatized glass slides. The lectin binding experiments showed that carbohydrates with different structural features on the surfaces exhibited strong and specific binding toward lectins. This demonstrates that carbohydrate chips are feasible for investigating protein-carbohydrate interactions in the post-genomic era.

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L088
DESIGN AND SYNTHESIS OF CARBOHYDRATE FUNCTIONALIZED NANOPLATFORMS TO PROBE VIRAL ADHESION

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Viral adhesion and infection is mediated through the binding of viral glycoproteins to cellular glycolipid rafts engaged in multivalent interactions. Previous studies have shown that the local environment and presentation geometry of receptor

binding sites are critical for maximal recognition. The actual binding sites in many polyvalent interactions have the dimension of several angstroms to tens of nanometers and the separation between neighboring sites also ranges from angstroms to hundreds of nanometers. An effective and reliable way to study and regulate these bioprocesses is to control polyvalent interactions through the positioning of glycolipids with nanometer precision. The local environment of these ligands can be engineered to effectively mimic their native states. Introduction of viral proteins to these nanoplatforms and monitoring of the binding process using microscopy and spectroscopy allows in situ determination of binding strength, stoichiometry, cooperativity, and kinetics in real time. These combined studies provide important insight into the mechanism of polyvalent protein/carbohydrate recognition at a molecular level. Understanding the mechanism of viral/host cell adhesion and recognition may ultimately lead to novel strategies for detecting and deactivating viruses in the environment.

L089
GLYCONANOPARTICLES AS HIGH AFFINITY PROBE FOR TARGETED PROTEIN ENRICHMENT AND IDENTIFICATION

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The interactions of glycoproteins and glycolipids on cell surface play important roles in cell-cell communication, proliferation and differentiation. Thus, study of carbohydrate-related interactions may provide new insights into their biological roles and reveal new possibilities for drug development and detection methods. We report here a new approach by using carbohydrate-encapsulated gold nanoparticles (c-AuNPs) as affinity probes for efficient separation and enrichment of target proteins (PA-I and SLT IB). Identification of protein and carbohydrate binding epitope were revealed by MALDI-TOF MS analysis. Combining glyconanoparticle and mass spectrometry, the detection limit by this method was 0.78 nM of PA-I in 100 μL protein mixture. The multivalent interactions between c-AuNPs and target proteins were also investigated by using surface plasma resonance (SPR) study. The K_is of P^k-AuNPs with 4, 13 and 20 nm sizes for PA-IL were 10^{-9}, 10^{-10} and 10^{-11} M, respectively, while those for SLT-IB were 10^{-11}, 10^{-12} and 10^{-13} M, respectively. These results showed that the multivalent interactions between target proteins and P^k-AuNP were affected by particle size, linker length and binding model. Therefore, we have demonstrated the feasibility of c-AuNP for specific enrichment and isolation of target protein, and mapping the binding amino acids containing sequence. The profound affinity enhancement by global shape nanoparticles needs to be further explored.

L090
FIRST FULLY ACTIVE LIPOTEICHOIC ACIDS OF GRAM-POSITIVE BACTERIA
SYNTHESIS AND BIOLOGICAL PROPERTIES

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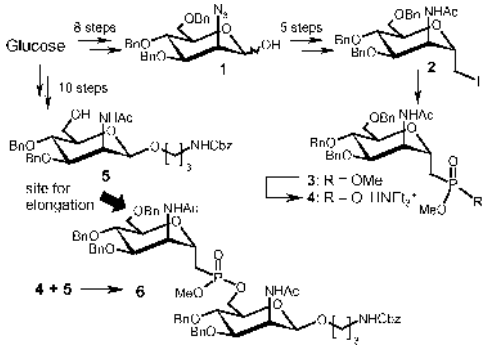
The inflammatory response to Gram-negative and Gram-positive bacteria can hardly be distinguished. However, while most of the responses to Gram-negative bacteria could be attributed to lipopolysaccharides (LPS) and their lipid anchor lipid A, no corresponding principle for Gram-positive bacteria was identified unambiguously during the last few decades. Lipoteichoic acids (LTAs) are found in most Gram-positive bacteria. Like LPS, LTAs are amphiphilic, negatively charged glycolipids. Yet, studies with commercial LTA preparations exhibiting significant activities could be traced back to contaminations with LPS. Only recently it was shown that these results are due to the preparation method employed for LTA: a novel, less harsh preparation method led to pure, biologically active LTA; a crucial role could be attributed to D-alanine residues at the polyglycerophosphate backbone of LTA, for instance of LTA of type I from *Staphylococcus aureus*.

To prove the biological activity of D-alanine residues containing LTA and to allow structure-activity analysis, *S. aureus* LTA was synthesized. The synthesis is particularly demanding, because the D-alanine residues are readily cleaved already at pH 8.5. The versatility of the synthetic concept is further demonstrated by its application to structural variations with the aim to elaborate the minimal structural requirement for biological activity. Also work on the structurally quite different *Streptococcus pneumoniae* LTA (type IV), having similar biological activities as *S. aureus* LTA, will be discussed.



L091
SYNTHESIS OF PHOSPHONO ANALOGUES OF FRAGMENTS OF N*EISSERIA MENINGITIDIS* TYPE A CAPSULAR POLYSACCHARIDE

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The resistance to *Neisseria meningitidis* (*N. m.*), a gram-negative bacterium causing meningitis, is mediated by the presence of antibodies directed against its capsular polysaccharides (CPS), which are associated with different serogroups. One of the most virulent is serotype A (Men A), whose CPS is constituted of (→6)-*N*-acetylmannosamine α-1-*P* as a repeating unit. The already existing vaccines against *N.m*. give a T-cell independent immune response, therefore inefficient in infants. The response can be improved by conjugation of the saccharidic portion to a protein carrier, which invokes T-cell involvement. In addition, anomeric phosphodiester bridges are chemically labile and therefore not appropriate for a vaccine. To overcome these problems, phosphonate 4 and dimer 6 have been synthesised as stable analogues of the repeating unit of Men A CPS. Key steps of the synthetic strategy are:

a) Synthesis of building blocks 1 and 5, the latter bearing a spacer at the reducing end suitable for conjugation to a proper protein carrier;
b) Introduction of dimethylphosphonate group through an Arbuzov reaction on iodide 2, obtained from 1 in 5 steps, and selective monohydrolysis of 3 to phosphonic acid 4;
c) Coupling of 4 with 5 *via* Mitsunobu reaction providing 6.

After removal of the protecting groups, compounds 5 and 6 have been evaluated through a competitive ELISA assay (IC_{50} values) by use of Men A antiserum. The promising results suggest that a remarkable biological activity is expected from higher oligomers, attainable by iteration of our protocol. Preliminary approaches towards this goal will be also discussed.

L092
COMBINATORIAL APPROACHES TO STUDY CARBOHYDRATE-PROTEIN INTERACTIONS

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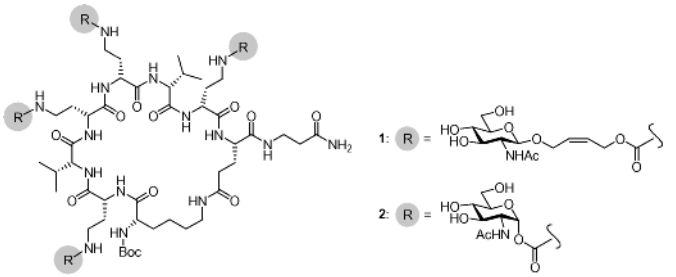
Multivalent carbohydrate-lectin interactions are the basis of numerous cell adhesion and signal transduction processes. The efficiency of a multivalent interaction is decisively dependent on the spatial presentation of the carbohydrates and the associated possibility to simultaneously occupy several binding sites of a multivalent lectin. We have established a combinatorial approach for probing the influence of the spatial presentation of carbohydrate epitopes on conformationally restricted scaffold molecules ("spatial screening")[1–3] comprising four steps: (i) split mix synthesis of a library of cyclic peptides, (ii) coupling of several copies of a carbohydrate residue to the peptide scaffolds in varying amounts and positions, (iii) on-bead immunosorbent lectin binding assay, (iv) identification of high-affinity ligands by single-bead micro sequencing. With this approach, we identified several high-affinity ligands for the model lectin wheat germ agglutinin (WGA), among them tetravalent neoglycopeptide **1**. Solution affinities to WGA were determined by an enzyme-linked lectin assay (ELLA). A good correlation between on-bead and solution affinities was found. Variation of the carbohydrate-peptide spacer led to tetravalent neoglycopeptide **2** which binds 15 times stronger to WGA than **1**. The data suggest that the spatial presentation of the GlcNAc residues on the cyclopeptide scaffolds is responsible for the high binding affinity.



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L093
A VERSATILE SYNTHETIC SYSTEM OF COMPLEX GLYCOPEPTIDES

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We have developed a high-throughput and versatile synthetic method of complex glycopeptides, composed of an accelerated solid-phase glycopeptide synthesis and enzymatic sugar elongation on water soluble polymer platform. To engage these chemical and enzymatic methods, two key technologies were developed as follows: 1) a microwave-assisted solid-phase glycopeptide synthesis (MA-SPGS) technology; 2) "molecular transporter" technology which can transport the full-length glycopeptide from resin to a water soluble polymer platform for subsequent enzymatic sugar elongation, and release of the resulted complex glycopeptides with a specific protease.
The MA-SPGS technology accelerated the reaction speed more than 10 times and improved the yield compared to common SPGS methods. The molecular transporter technology required only a simple purification to remove impurities and by-products, and allowed for enzymatic sugar elongation, and enzymatic release of the target products.
As a result, a high-throughput combinatorial glycopeptides synthesis could be achieved, and applied for constructing MUC1 related glycopeptide library.
This study was supported partly by New Energy and Industrial Technology Development Organization (NEDO).

L094
MODIFIED NUCLEOTIDE SUGARS AS NOVEL TOOLS FOR GLYCOBIOLOGY

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The paper presents our work on the chemo-enzymatic synthesis of novel modified nucleotide sugars and their potential use in glycobiology and biomedical research. It is well documented in literature that changes in the glycosylation pattern of glycoproteins are related to diseases like rheumatoid arthritis (IgG), IgA nephropathy (IgA) or Tn-syndrome (cancer). The aim of our current project is the development of a method for the specific labelling of the involved glycan structures by the glycosyltransferase-catalyzed transfer of modified donor substrates.
The combination of the enzymatic oxidation of UDP-Gal(NAc) using galactose oxidase with the chemical modification using (biotin-ε-amido-caproylhydrazide, BACH) in one-pot led to the efficient synthesis of UDP-6-biotinyl-Gal(NAc) in 100 mg scale.
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 potential of UDP-6-biotinyl-Gal as a novel donor substrate for human galactosyltransferases lies in the targeting of distinct acceptor structures, e.g. disease-related under-galactosylated N-and O-glycans of glycoproteins, In rheumatoid arthritis the N-glycans of IgG are characterized by an increased under-galactosylation. In the present study IgG from different species showing different levels of under-galactosylation were chosen as model structures for the galactosyltransferase (GalT)-mediated labelling with UDP-6-biotinyl-Gal. The successful transfer of 6-biotinyl-Gal was demonstrated by a sandwich Enzyme-Linked-Streptavidin-Assay (ELSA).
Work is in progress to optimize transfer conditions for the GalT onto IgG and to establish a diagnostic assay for under-galactosylated IgG from the serum of patients with rheumatoid arthritis.

L095
A NOVEL MICROWAVE ASSISTED GLYCOSYLATIONS USING METHYL GLYCOSIDE DONORS

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Efficient microwave-assisted glycosylations from methyl glucopyranosides will be presented.
Glycosylation has been well studied especially over the past quarter century. In general, alkyl glycosides are not suitable for glycosyl donors because they require harsh acidic conditions to break the glycosyl bonds, with the exception of 4-pentenyl glycosides which are able to be activated in intramolecular systems. In order to form new glycosyl bonds, therefore, glycosyl donors have efficient leaving groups at their anomeric centers. Although methyl glycosides are often used as protecting groups for the anomeric center, they *never* act as glycosyl donors in the formation of new glycosyl linkages except in a case of the report from Mukaiyama *et al*. which has required silyl-protection for acceptor hydroxyl groups to achieve higher glycosylation yields.
Despise these historical backgrounds, we have found a new glycosylation system that with microwave irradiation has lead to newly glycosylated compounds, allowing methyl glucopyranosides as donors, as simple to exchange glycosyl bonds, eg. 88% yield α:β = 57:31. We will present the effects of microwave irradiation on this unique glycoside exchanging reaction from some view points such as amount of Lewis acid promoters and acceptors, hydroxyl protecting groups of

methyl glucopyranosides donors for reactivity and neighboring effect. Also, applications for oligosaccharide synthesis and preparing libraries will be presented.

L096
SYNTHESIS AND CHOLERA TOXIN BINDING OF NOVEL CALIXARENE-BASED GLYCOCONJUGATES

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Multivalency is a phenomenon often used by nature to strengthen the binding in cases where the single ligand-receptor interaction is weak. When saccharides are involved, this phenomenon is also called the "glycoside cluster effect". The design and synthesis of multivalent (neo)glycoconjugates, able to interfer in these recognition processes, is a challenging topic in bioorganic and supramolecular chemistry. The bacterial AB_5 toxins, *e.g.* cholera toxin (CT), represent peculiar examples of proteins involved in multivalent interactions with their biological substrates, such as the GM1 ganglioside. In this context, we are developing a series of polyvalent glycomimetics based on the conjugation between calix[n]arene scaffolds and a variable number of a *pseudo*GM1 unit, which was previously reported as a high affinity ligand for CT. Our aim is to obtain efficient inhibitors and get more information on multivalency, not yet completely understood in all its aspects. Fluorescence titration experiments and Elisa tests evidenced that the divalent derivative binds to CT (concentration at 50% of saturation = 48 nM) more efficiently than the monomeric *pseudo*GM1 (K_d=190 μM) and slightly better than the natural GM1 oligosaccharide (concentration at 50% of saturation = 219 nM). We are trying to confirm these results by using other techniques, in particular Surface Plasmon Resonance, and are extending the investigation to the other members of the calixarene series, also with the aim to disclose the role of the different neoglycoconjugates structural parts and of the glycoside cluster effect in CT recognition.

L097
HIGHLY SELECTIVE GLYCOSYLATED PRODRUGS OF TOXOFLAVIN GLYCOSIDES FOR ANTIBODY-DIRECTED ENZYME TUMOR THERAPY

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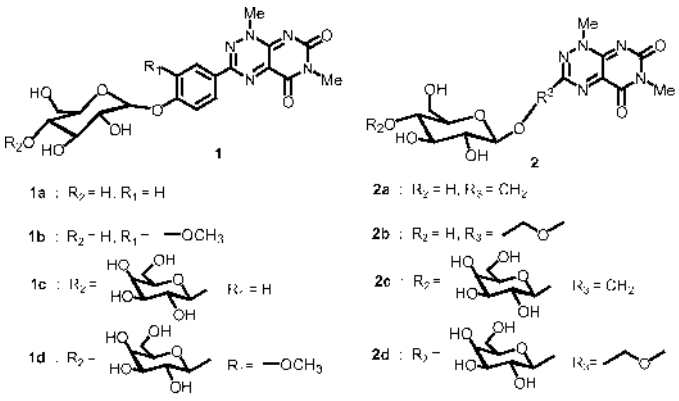
Since toxoflavin**,** a naturally occurring antibiotic, was isolated in 1933 from *Pseudomonas cocovenenans*, the 7-azapteridines have been the subject of a great deal of synthetic and biological study. Recently this compound has stimulated considerable interest because of its high cytotoxicity and potent anti-tumour activities. To decrease the toxicity and increase the anti-cancer activity of these cytotoxic agents, we developed new potent prodrugs of toxoflavin glycosides **1a-d** and **2a-d** which can be used in antibody-directed enzyme prodrug therapy (ADEPT). These prodrugs showed a remarkable selectivity with a $Q(IC_{50\ drug}/IC_{50\ prodrug})$ value of up to more than 1500. The formed drugs have high potency against human colon carcinoma cells with IC_{50} of 1 ~ 10 nM.



L098
SYNTHESIS OF NOVEL NOJIRIMYCIN DERIVED OXAZOLIDINONES AND OXAZINANONES

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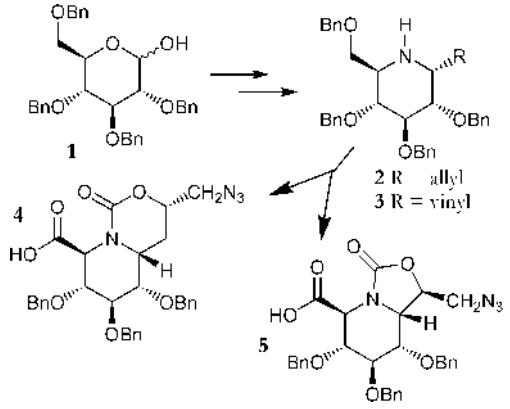
Polyhydroxylated nitrogen heterocycles, usually referred to as iminosugars, are known for their inhibitory activity toward carbohydrate-processing enzymes, suggesting their use in a wide range of therapeutic applications, such as treatment of diverse viral infections, human immunodeficiency virus (HIV), human hepatitis, bovine viral diarrhea virus (BVDV), Japanese encephalitis virus and dengue virus, as well as cancer, diabetes, tuberculosis, malaria and lysosomal storage diseases. Due to the tremendous potential of these molecules as invaluable tools in the study of the biological functions of oligosaccharides, and their often potent inhibitory activity toward carbohydrate-processing enzymes, a variety of

monocyclic and bicyclic iminosugars have been synthesised or isolated from natural sources. In a project devoted to the synthesis of iminosugar azido acids, novel nojirimycin-derived bicyclic structures, containing a 1,3-oxazolidin-2-one and a 1,3-oxazinan-2-one moiety have been synthesised. 1,3-Oxazolidin-2-ones and 1,3-oxazinan-2-ones are important target molecules in organic synthesis because of their pharmacological properties. Oxazolidinones can act as antibiotics against highly resistant Gram-positive bacteria, while 1,3-oxazinan-2-ones are important heterocycles present in several biologically active natural products such as maytansine and its analogues, investigated as anti-cancer drugs.

The key steps of the synthesis are first the formation of the nojirimycin ring from commercially available 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranose (1), and secondly the formation of the oxazinanone ring to derivative (4), or oxazolidinone ring to derivative (5).



L099
CARBOHYDRATE – CARBOHYDRATE INTERACTION AS A MAJOR FORCE INITIATING CELL RECOGNITION

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The first clear evidence that cells can recognize each other came 1907 from H. V. Wilson showing that dissociated marine sponges will re-associate species specifically. Such cell-cell recognitions are mediated via circular and linear surface proteoglycan-like molecules of Mr $2x10^7$, requires sea water Ca^{++} concentrations and early data pointed to a carbohydrate involvement. Proteoglycan-coupled beads sorted out species-specifically like live sponge cells. Species-specific carbohydrate epitope mAbs inhibited species-specific sponge cell recognition and adhesion. Glycan coated beads of the same species aggregated. This still left the possibility open that the recognition process is mediated via protein-carbohydrate interactions. Evidence that the glycan portion alone mediated the species-specific proteoglycan recognition came only forth when glycan coated cell-sized beads sorted out exactly as live sponge cells. AFM between the same species glycans revealed forces in the 300 picoNewton range. Significantly higher than between different species. Remains the question whether CCI between sponge proteoglycans is the mechanism mediating the recognition and the force between live cells. Support for this concept comes from 3 experiments: when live cells were pre-incubated with mAbs against the species-specific carbohydrate epitope (Glc*p*NAc3S(ß1-3)Fuc*p*), such cells did not bind anymore to beads coated with the same glycan. Furthermore, binding of adult live sponge cells to glycan coated plastic surfaces could be inhibited species-specifically with the same carbohydrate-directed mAbs. And finally, live sponge larval cells, for which the proteoglycan recognition is critical, also can be inhibited with the carbohydrate-directed mAbs from binding to their species-specific-glycan coated plastic plates.

L100
GLYCONANOTECHNOLOGY APPLIED FOR UNDERSTANDING CARBOHYDRATE-CARBOHYDRATE INTERACTIONS

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Carbohydrate-carbohydrate interaction is a reliable and versatile mechanism for cell adhesion and recognition. Glycosphingolipid (GSL) clusters at the cell membrane are mainly involved in this interaction. To investigate carbohydrate-carbohydrate interaction an integrated strategy (Glyconanotechnology) was developed. This strategy includes both polyvalent models mimicking GSL clustering at the cell membrane and analytical techniques to evaluate the interactions. The results obtained by means of this strategy and current status are presented.

To create polyvalence we designed two simple systems, based on self-assembled monolayers (SAMs), by which neoglycoconjugates of the natural epitopes are attached to bi-dimensional (2D) and tri-dimensional (3D) gold surfaces creating in this way multivalent array of carbohydrates mimicking GSL patches at the cell surface. The polyvalent 3D model systems are based on sugar modified gold nanoclusters. The so-called glyconanoparticles (GNPs) provide a glycocalyx-like surface with globular carbohydrate display and chemically well-defined composition to study carbohydrate interactions and to interfere cell-cell adhesion processes. The two models are highly complementary and the use of both of them to measure biologically relevant

interactions demands the application of new surface and solution techniques.
The combination of these 2D and 3D polyvalent model systems together with classical analytical techniques such as weak affinity chromatography (WAC), isothermal titration calorimetry (ITC) and other techniques as transmission electron microscopy (TEM), atomic force microscopy (AFM), or surface plasmon resonance (SPR) allowed us to demonstrate and to quantify Ca^{2+}-mediated carbohydrate-carbohydrate interactions.

Reference
J. M. de la Fuente and S. Penadés, Glycoconjugate J., 21, 149-163, 2004

L101
ANALYSIS OF GM3-Gg3 INTERACTION USING CLUSTERED GLYCOCONJUGATE MODELS CONSTRUCTED FROM GLYCOLIPID MONLLAYERS AND ARTIFICIAL GLYCOCONJUGATE POLYMERS

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We proposed model systems consisting of clustered GM3 (NeuAcα2-3Galβ1-4Glcβ1-1'Cer) on Langmuir monolayer and clustered Gg3 trisaccharide (GalNAcβ1-4Galβ1-4Glcβ1-NHCO-) along a polystyrene chain to investigate carbohydrate-carbohydrate interactions by surface pressure-area (π-A) isotherms and surface plasmon resonance (SPR).
The π-A isotherm of the GM3 monolayer was expanded substantially and specifically by the Gg3-trisaccharide-bearing glycoconjugate polystyrene [PN(Gg3)] even at 10^{-12} M. The PN(Gg3)-induced expansion of the GM3 monolayer required no calcium ion, and the expansion was strongly inhibited in the presence of urea and acetamido sugars. SPR studies of the GM3-Gg3 interaction were carried out to estimate the affinity constant and specificity of the interaction quantitatively. PN(Gg3) was adsorbed onto the GM3 monolayer strongly and specifically with an apparent affinity constant of $K_a = 2.5 \times 10^6\ M^{-1}$.
We discuss the mechanism of the GM3-Gg3 interaction on the basis of the relationship between affinity and structure from the following viewpoints.

(1) Contribution of each structural unit in GM3-Gg3 interaction.
(2) Driving forces of GM3-Gg3 interaction.
(3) Effect of Ca^{2+} Ions.

We have found that the NHAc groups of *N*-acetylneuraminic acid in GM3 and of GalNAc in Gg3 play an important role in the GM3-Gg3 interaction and that PN(Gg3) recognizes not only some specified portions of GM3 but also the trisaccharide as a whole.

Glycoconjugate J., **21**, 139–148 (2004).

L102
A GLYCOSYNAPSE IN MYELIN?

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Myelin, the multilayered membrane which surrounds nerve axons, is the only example of a membranous structure where contact between extracellular surfaces of membrane from the same cell occurs. The two major glycosphingolipids (GSLs) of myelin, galactosylceramide (GalC) and its sulfated form, galactosylceramide I^3-sulfate (SGC), can interact with each other by trans carbohydrate-carbohydrate interactions across apposed membranes. They occur in detergent-insoluble membrane domains containing kinases and thus may be located in membrane signaling domains. These signaling domains may contact each other across apposed extracellular membranes, thus forming "glycosynapses" in myelin. Multivalent forms of these carbohydrates, i.e., GalC/SGC-containing liposomes, or galactose conjugated to albumin, have been added to cultured oligodendrocytes (OLs) to mimic interactions which might occur between these signaling domains when OL membranes or the extracellular surfaces of myelin come into contact. These interactions between multivalent carbohydrate and the OL membrane cause co-clustering or redistribution of myelin GSLs, GPI-linked proteins, several transmembrane proteins, and signaling proteins to the same membrane domains. They also cause depolymerization of the cytoskeleton, indicating that they cause transmission of a signal across the membrane. Their effects are similar to those of anti-GSL antibodies on OLs, shown by others, suggesting that the multivalent carbohydrate interacts with GalC/SGC in the OL membrane. The actin filament stabilizing reagent, jasplakinolide, prevented all of these effects indicating that depolymerization of actin microfilaments was an early event and necessary for all other effects. These effects were also prevented by several kinase inhibitors and the phosphatase inhibitor, tautomycin, providing clues concerning the signal transduction pathways involved. Communication in both directions between the myelin sheath and the axon regulates both axonal and myelin function and is necessary to prevent neurodegeneration. Participation of transient GalC and SGC interactions in glycosynapses between the apposed extracellular surfaces of mature myelin might allow transmission of signals throughout the myelin sheath and thus facilitate myelin-axonal communication. (Supported by the Multiple Sclerosis Society of Canada).

L103
UNRAVELLING THE BASICS OF THE CARBOHYDRATE-CARBOHYDRATE SELF-RECOGNITION IN MARINE SPONGE CELLS

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The species-specific cell adhesion of the marine sponge *Microciona prolifera* involves proteoglycan-like macromolecular complexes otherwise known as aggregation factors (MAF). In a Ca^{2+}-independent process, a 6 kDa glycan located on MAF arms (known as g6) adheres to cell surface receptors, while a 200 kDa glycan protruding from the MAF central ring (known as g200) promotes cell adhesion via a Ca^{2+}-dependent self-association process. One of the carbohydrate epitopes involved in the g200 self-association is the sulfated disaccharide β-D-Glc*p*NAc3*S*-(1-3)-α-L-Fuc*p*.
The sulfated disaccharide was synthesized with an amino spacer at the reducing end and attached multivalently to BSA via a linker system. Using surface plasmon resonance (SPR), it could be demonstrated that the disaccharide-BSA conjugate (immobilized) / disaccharide BSA conjugate (analyte) system can mimic the g200 – g200 interactions in the presence of Ca^{2+} ions. No interaction was seen in the presence of Mg^{2+} or Mn^{2+} ions. More detailed proofs were obtained by transmission electron microscopy (TEM) of water-soluble gold glyconanoparticles (GNPs) multivalently coated with the thiol-spacer-containing sulfated disaccharide, and by atomic force microscopy (AFM) using flat gold-surface samples and gold cantilevers functionalized with self-assembling monolayers of the thiol-spacer-containing disaccharide. Also here Mg^{2+} ions were not able to initiate the interaction, but Cd^{2+} ions turned out to be good initiators. TEM studies of GNPs decorated with variants of the sulfated disaccharide indicated that the fucose methyl group and the glucose 3-*O*-sulfate and 2-*N*-acetyl groups are essential in the self-recognition.

L104
LEWIS X - LEWIS X RECOGNITION: A HIGHLY SPECIFIC INTERACTION INVOLVED IN CELL ADHESION

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Although rarely considered in the past, carbohydrate-carbohydrate specific interaction has recently emerged as a potentially important interaction in cell adhesion processes. One calcium mediated homotypic recognition between two Lewis x determinants has been proposed to drive cell adhesion in murine embryogenesis. By using synthetic neoglycolipids bearing this carboyhydrate to make giant vesicles, and micropipet aspiration technique to measure their adhesion strength, the existence of this specific interaction was confirmed. However, the neoglycolipids allowed a strong orientational freedom of the Lewis x group unlike the natural glycolipid in which the Lewis x group orientation is strongly constrained. The natural lipid was then synthesised and vesicles adhesion measured with the same micropipet technique. A stronger adhesion energy was measured showing that the restricted orientation of the Lewis x group enhanced (five-fold) the adhesion. Control experiments were made by replacing the Lewis x by Lewis a on one vesicle surface. In the Lewis a, the galactose and fucose are permutated relative to the Lewis x, which leaves non-specific interactions identical for both compounds. This underscored the high sensitivity of the Lewis x - Lewis x recognition to molecular structure. Along with studies from other groups, these results illustrate the wealth of specific interactions that carbohydrates can provide through their wide variety of structures and spatial orientations.

L105
CARBOHYDRATE-CARBOHYDRATE INTERACTION (CCI) AS INITIAL DRIVING FORCE FOR A VARIETY OF *CIS* AND *TRANS* CELLULAR INTERACTIONS

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Molecular interactions supporting cell-to-cell adhesion (*trans* interaction) are basically redundant processes, as in selectin/ ICAM-1-mediated neutrophil adhesion to endothelial cells (ECs). CCI is a rapid process, followed by the slower, stronger process of protein-to-protein interaction (PPI). Examples are: (a) B16 cell adhesion to mouse ECs during metastasis, mediated by rapid binding of GM3 (on B16) to LacCer (on ECs), followed by slow α4β1 interaction with VCAM-1 (1,2); (b) rapid Ca^{2+}-dependent autoaggregation of mouse F9 cells or compaction of morula-stage cells. Both F9 and morula express Le^x glycan. Non-Le^x-expressing variant PYS-2 cells do not show autoaggregation. Le^x glycan *per se* displays Ca^{2+}-dependent self-recognition (3). This process is redundant with E-cadherin-mediated PPI.
In contrast to *trans* interaction as above, there are numerous molecular interactions within the same cell surface plane (*cis* interaction). Integrins and growth factor receptors (GFRs) with or without tetraspanins are clustered within the microdomain, which we term "glycosynapse". Functions of integrins and GFRs are strongly affected by state of receptor N-glycosylation and by types of surrounding ganglioside within glycosynapse (4). We studied possible interaction between N-linked glycans and GM3, focused on EGF receptor

function, since EGF tyrosine kinase is inhibited by GM3. GM3 interacts strongly with hybrid-type N-linked glycans having multiple GlcNAc. Implications of these findings will be discussed.

***References**: (1) Kojima, JBC 267: 17264, 1992. (2) Sadahira, In Vitro 30A: 648, 1994. (3) Kojima, Glycoconj J 11: 238, 1994. (4) Hakomori, PNAS 99: 225, 2002.*

L106
ANALYSIS OF OLIGOSACCHARIDE METABOLISM IN SINGLE CELLS

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Single cell analysis provides information on cell-to-cell variation in populations of cells or on individual steps in cellular development. It is useful for analysis when sample sizes are limiting such as in biopsies or for confirmation of deleterious gene deletions. An overview of two methods to monitor oligosaccharide metabolism in individual cells will be given.

In the first approach, cells are grown in the presence of fluorescently labelled oligosaccharides, which are taken up into cells and modified by the action of glycosidases and glycosyltransferases. This requires the disruption of individual cells after compound uptake and metabolic conversion, followed by the analysis of the entire cellular contents by capilliary electrophoresis with laser induced fluorescence detection (CE-LIF). This has been employed to monitor βGal(1,4)GlcNAc (LacNAc) inter conversions in HT29 cells and α-glucosidase I and II activities in yeast. In the second method, a single cell is lysed in 100-200 nL of buffer containing fluorescently labelled oligosaccharide substrates. Aliquots (5 nL) are removed with a nanopipettor and diluted for analysis by CE-LIF. This technique has been employed to monitor the activities of α-glucosidase I, II and blood group A glycosyltransferase in individual cells and to confirm knock out of the α-glucosidase I gene in *Arabidopsis* embryos. In this method, the progress of oligosaccharide modification can be monitored as a function of time.

L107
LECTIN ARRAY-BASED ANALYSIS OF PROTEIN GLYCOSYLATION

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Glycoproteins produced by mammalian cells are a mixture of glycoforms of the same protein with differing patterns of glycans. Most factors that influence cell growth influence the glycoform mixture produced by the cells. Because glycosylation is not a template-guided process, and because glycans are diverse branched structures, the analysis of glycoforms remains one of the greatest challenges to the study and production of glycosylated proteins and to understanding the role of glycosylation. Procognia has developed lectin array and algorithm based technology to structurally analyze the glycans on intact, unpurified glycoproteins. In the analysis, glycans on the proteins bind to the lectin array and are detected by labeled probes. Probes can be antibodies directed against the protein core or lectins that recognize other glycans on the same protein. A proprietary knowledge base of lectin-glycan recognition behavior and proprietary algorithms are used to deconvolute a fingerprint, a histogram of normalized lectin binding data, into a quantitative analysis of glycan structures present in a glycoform mixture. Multiple replicates and concentrations of each arrayed lectin, internal calibration standards and controls result in highly reproducible data. The assay is rapid and automated; up to 20 samples can be analyzed in parallel with controls in less than 4 hours. The analysis can be performed directly in culture medium with nanomolar to low micromolar quantities of starting sample. These features allow the correlation of glycosylation with biological properties of a glycoprotein.

L108
SOLID-PHASE OLIGOSACCHARIDE TAGGING (SPOT): EARLY RESULTS VALIDATE A NEW TECHNIQUE FOR LABELING AND MANIPULATING GLYCANS.

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Glycan chains released from glycoproteins or glycolipids are frequently chemically tagged at the reducing end prior to analysis by HPLC, CE or mass-spectrometry. We report here a simple method to accomplish such labeling that represents an important advance in the general strategy termed "Glycoblotting" (Nishimura et al., Angew. Chemie Intl. Ed. 117 (2005) 93-98).

In the SPOT process described here, hydroxylamine groups are attached to particles (CPG or PEGA beads) through optional spacer-chains containing a cleavable linker. After covalent capture of a reducing sugar and washing, the excess unreacted hydroxylamine groups are capped, the oxime double bond is reduced and the resulting sugar-NH- group is reacted with a derivatizing agent. All reagents can be used at high concentrations as the excess can be washed away while

the glycan remains concentrated on the beads. There can be no selective loss during the manipulations.
The SPOT process has been optimized using lacto-N-tetraose. Labels are added using aryl isothiocyanates yielding fluorescently tagged glycans (FITC and TRITC) for CE analysis, bromine-containing labels to facilitate analysis by MS or stable isotope-labeled reagents to permit differential Glycomics analysis by MS. The immobilized labeled glycans can also be made fully accessible to glycosidases permitting enzyme-assisted sequencing on CPG. The clear benefits of the SPOT method are the simplicity of the manipulations, the ability to prepare several different derivatives of the immobilizd glycan within a couple of hours and the release of a product of high purity into a small volume for further analysis.

L109
COMPLEMENTARY MALDI-Q/TOF AND MALDI-TOF/TOF MS/MS FEATURES FOR *DE NOVO* SEQUENCING OF COMPLEX GLYCANS

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Concerted MALDI-MS profiling and MS/MS sequencing of the permethyl derivatives of released glycans is arguably the single most informative and conducive mean of mapping a glycome. Recent coupling of MALDI source to Q/TOF and later TOF/TOF types of mass spectrometers has afforded high sensitivity and quality CID-MS/MS capability for *de novo* glycan sequencing. Based on a wide range of glycans analyzed, we demonstrate here the fundamental difference between the complementary fragmentation patterns afforded by the Q/TOF versus TOF/TOF and how they can be effectively used in concert to solve real structural problems. On one hand, multifaceted sequencing of permethylated, sulfated or phosphorylated N-glycans are highlighted in conjunction with a strategic workflow which incorporates judicious sample pre-fractionation and optimized chemical derivatization steps. On the other hand, focus is directed towards facile definition of the branching pattern of large glycans including the commonly found poly-N-acetyllactosaminoglycans, with and without further glycosyl substitution. Low energy CID-MS/MS of sodiated parents on the Q/TOF tends to induce multiple cleavages which could be turned into advantages for distinguishing branching, core structure and multiple substitutions by virtue of the *O*-Me tag. In contrast, MS/MS by MALDI-TOF/TOF predominantly results in a single cleavage event, yielding a certain complement of ions which reflects the branching nature and sites. Ring and concerted double cleavages could be preferentially induced over glycosidic cleavages by elevated collision cell energy to give linkage information. These knowledge-based features are exploited to define the highly branched polylactosaminoglycans from rabbit erythrocytes and to identify similar structures elsewhere.

L110
TOWARDS AUTOMATED ANALYSIS OF LARGE POOLS OF GLYCOPROTEINS COMBINING MASS SPECTROMETRY AND *IN SILICO* WORKFLOWS

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Glycoproteins are abundant and crucial for unlimited numbers of biological processes. The analysis of glycoproteins has so far been tedious and could have been performed only for one glycoprotein at a time. The current large scale analysis depended on separating glycans and peptides from original glycoproteins followed by separate analysis. However the major problem in here is the loss of information of which glycans are linked to which amino acids and thus failure to reconstruct the original structure of the glycoprotein. We have initiated a project to analyse the structures of glycoproteins from human plasma. Starting from a plasma specimen, we first run HPLC and fractionate it into aliquots containing only tens of glycoproteins each. After trypsination we run the mass spectrometry analysis of each aliquot and with the help of our newly developed proprietary software we can identify from each HPLC fraction:
a) the proteins involved
b) the glycopeptides produced by trypsination
c) the glycosylated N- and O-sites on these glycopeptides
d) the monosaccharide composition from the glycopeptides
e) the putative glycan structure separately to each site
Taken together we can now tell which glycoproteins were present in the original mixture, characterize their glycosylated sites and the glycans decorating these sites. All this can be done in a semi-automated manner from HPLC-fractions each containing >10 original glycoproteins and now allows us to analyse and identify the structure of up to hundreds of glycoproteins.

L111
CHARACTERIZATION OF ANOMERIC SIALO-CONJUGATES USING FAB-MS/MS --- DISTINCTION BETWEEN α– AND β–GLYCERO-TYPE GANGLIOSIDES

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FAB-MS/MS spectra of 1-*O*-octadecyl-3-*O*-*N*-acetylneuraminyl- *sn*-glycerol sodium salt anomers were studied. Those spectra indicate that the distinction between α- and β-anomers is possible and the fragmentations are accounted for on the basis of the individual configuration with a specific conformation required for the β-anomer. The method seems to be versatile when the sialylated moiety is large.
In the (+)-ion mode, the most prominent product ion of $[M'+Na]^+$ was the sodiated sodium 2,3-ene-sialate (*m/z* 336.1) for the α-anomer, but the sodiated sodium alkoxyglycerol (*m/z* 389.3) for the β-anomer. We assume that all side-chains of the α-anomer are equatorial as in the natural pyranose 1C-conformation, giving the expected sugar-side product ion. In the β-anomer, the large glycerolipid moiety disfavors the axial conformation, thus the ring flips to take C1-conformation. Then, the dissociated carboxylate anion approaches and abstracts the 4-hydroxyl proton to form a free carboxylic acid. Thus liberated Na^+ may exchange the lipid's *sn*-2-OH, producing the sodium adduct of the sodium *O*-octadecylglyceroxide. On the (–)-MS/MS of $[M'–Na]^-$, the α-anomer showed the most intense ion at *m/z* 290.0 (2,3-ene-sialate anion), while the β-anomer showed a prominent ion at *m/z* 142.1. Our hypothesis is that the β-anomer has an additional route to this ion (C4-C7), which is different from the common *m/z* 142 (C2-C5). Again, proximity of the carboxylate to the 4-OH is required.

L112
ADVANCED ANALYTICAL SYSTEMS FOR THE BINDING INTERACTION OF STRUCTURALLY DEFINED OLIGOSACCHARIDES WITH PROTEINS/CELLS: USE OF SURFACE PLASMON RESONANCE (SPR) OR GOLD NANO-PARTICLES (GNP)

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SPR is a very powerful tool for the real-time study of the specific interactions between biological molecules without labeling. We developed an advanced method for the immobilization of oligosaccharides onto a gold-coated chip for SPR. Linker compounds containing thioctic acid were designed. A synthetic, structurally defined disaccharide in heparin was incorporated to the linker and thus immobilized as a ligand for SPR, and the binding interaction with von Willebrand factor (vWF), its known heparin-binding domain, and integrins were quantitatively analyzed. Also various oligosaccharides were similarly immobilized on chips and their interaction with lectin proteins were systematically evaluated. The bound proteins on the chip were further analyzed with MALDI-TOF/MS.
To establish an on-site analytical tool, the above immobilization technique was applied to GNP. For example, a ligand-conjugate containing α-D-glucopyranoside was prepared using the linker, and was then reacted with GNP, followed by dialysis to prepare α-D-glucopyranoside immobilized GNP (Glcα-GNP). The color of Glcα-GNP in PBS was purple, showing plasmon absorption at 520 nm. When Glcα-GNP was incubated with Con A, the colloid solution became colorless as nano-particles were quickly aggregated. The change was detected visually and the rate of change was dependent on the concentration of Con A. The dissociation constant was calculated to be 940 nM, which was similar to that obtained by SPR using the same ligand-conjugate. The aggregate was dissolved by adding excess D-glucose or 1 M HCl, and the Con A in the original solution was quantitatively recovered, offering a quick purification of protein.

L113
A NOVEL STRATEGY FOR INTELLIGENT IDENTIFICATION OF OLIGOSACCHARIDES USING OBSERVED AND SIMULATED MS^n SPECTRAL LIBRARIES

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Glycomics lags far behind proteomics because of the difficulties arisen from their structural complexities such as the variations of branching, linkage and stereo-chemistry. Recently, tandem mass spectrometry has been revealing that oligosaccharides might have characteristic fragment patterns. However, no practical method for glycan structural analysis with a wide range applicable to glycomics currently exists. We describe here a strategy for the rapid and accurate identification of the oligosaccharide structures using only mass spectrometry. It is based on a comparison of the observed multi-stage tandem mass spectra between the analyte and the structurally defined oligosaccharide library prepared by human glycosyltransferase library (1). CID spectra of these oligosaccharides were acquired with MALDI-QIT-TOF mass spectrometer. For smart identification, we developed the intelligent MS^n acquisition protocol, where a computer suggests the next parent ion which should yield the most informative spectrum with reference to the spectra in the library. Using this strategy, we could identify the structure of N-glycans in immunoglobulin G as examples.

Simulation of the fragmentation patterns for any given structures is desired as another powerful approach. Using UDP-$^{13}C_6$-D-galactose, the sets of biantennary, triantennary, and tetraantennary N-glycans which carried the $^{13}C_6$-galactose at complementary positions were enzymatically synthesized. We prepared the fragmentation templates based on the accurate assignment of the CID spectra of these oligosaccharides. Using these templates, we simulated the MS/MS spectra for 33 oligosaccharides to store the simulated spectral library. As a demonstration, the biantennary N-glycan containing a Lac-diNAc residue was successfully identified by matching with the simulated MS/MS spectral library.

Reference

1. A. Kameyama et al. (2005) Anal. Chem., in press.

L114
MASS SPECTROMETRIC STRATEGIES FOR THE N-GLYCOSYLATION ANALYSIS OF SERUM GLYCOPROTEINS IN PATIENTS WITH CONGENITAL DISORDERS OF GLYCOSYLATION (CDG) AND GALACTOSEMIA.

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Congenital disorders of glycosylation (CDG) and galactosemia are inherited metabolic diseases which have in common the occurrence of an abnormal glycosylation pattern of some serum glycoproteins. CDG are due to defects in the synthesis of N-glycans and less frequently affect the O-glycosylation pathway. As glycoproteins are ubiquitous molecules, CDG present as multisystemic disorders with a prominent central nervous system involvement and a variable clinical spectrum. CDG type I defines defects owing to impaired synthesis of the lipid-linked oligosaccharide and/or its transfer to the nascent glycoprotein in the cytosol and in the endoplasmic reticulum, CDG type II refers to abnormalities in the glycoprotein processing at the Golgi level. The most common form of galactosemia, due to a deficiency of galactose-1-phosphate uridyltransferase (GalT), causes accumulation of galactose and galactose-1-phosphate in blood and tissues, and if untreated, produces severe symptoms as mental retardation, cirrhosis of the liver, and cataracts. In both CDG and galactosemia an abnormal glycosylation pattern involving multiple serum glycoproteins may be promptly detectable by the isoelectric focusing (IEF) of serum proteins following by immunodetection of transferrin isoforms which show a cathodal shift due to the absence of terminal negatively charged sialic acid residues. Although IEF allows the measurements of the sialylation degree in serum transferrin, it is uninformative about the presence of isoforms due to the partial occupancy of one or both transferrin glycosylation sites and the detailed analysis of the N-glycan structures. In order to identify glycosylation abnormalities both in CDG and in galactosemia patients, additional techniques such as matrix assisted laser-desorption ionisation (MALDI) and Electrospray mass spectrometry may be essential. We used mass spectrometry based strategy to define the glycosylation degree of intact glycoproteins and to detect changes in serum protein N-glycan profiles. These findings open the way to a better understanding of the biochemical mechanisms of defective glycosylation.

L115
STRUCTURE/FUNCTIONAL STUDIES OF TRYPANOSOMAL SIALIDASES

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The intracellular parasite *Trypanosoma cruzi*, etiologic agent of Chagas' disease, sheds a developmentally regulated surface trans-sialidase (TcTS), which is involved in key aspects of parasite-host cell interactions. Although it is homologous to viral and bacterial sialidases, TcTS behaves as a highly efficient sialyl-transferase not dependent on a sialyl-nucleotide donor substrate and barely inhibited by known sialidase inhibitors. We have followed a multidisciplinary approach, involving protein crystallography, site-directed mutagenesis, synthetic chemistry and mass spectroscopy, in order to advance in the understanding of substrate recognition and catalytic mechanism at the atomic level. High resolution (1.6 Å) structures of TcTS have been solved, corresponding to the enzyme alone, in complex with substrates and also, kinetically trapped in the intermediate state, covalently bound to the sialyl moiety. These 'snapshots' of the catalytic cycle, together with the structure of TrSA, the sialidase from *T. rangeli* (which albeit being 70% identical, performs a strict hydrolytic activity), allowed us to extract functional information subsequently validated by mutagenesis. Although both enzymes operate through a ping-pong mechanism, by using a rather exceptional residue as the nucleophile (tyrosine 342), several mutations in the periphery of the reaction center account for the novel trans-glycosidase activity of TcTS. A substrate-triggered conformational rearrangement only observed in TcTS seems to be the key event leading to a much better sialic acid-acceptor binding site and overall water-exclusion, resulting in high trans-glycosidase efficiency. These trypanosome enzymes illustrate how a glycosidase scaffold can achieve efficient glycosyltransferase activity critical for the parasite survival and provide a framework for ongoing structure-based inhibitor design.

L116
MOONLIGHTING FUNCTIONS OF THE *TRYPANOSOMA CRUZI* TRANS-SIALIDASE

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Given the enormous complexity of vertebrates, their genome is relatively small compared with bacteria and single-celled eukaryotes. One explanation is that the functions of many proteins derived from a given gene can be expanded by various mechanisms such as moonlighting activities. The paradigm moonlighting protein is phosphoglucose isomerase (PGI), which converts glucose 6-phosphate to fructose 6-phosphate in the glycolytic pathway of the metabolism. In addition, PGI moonlights as a pleiotrophic cytokine independently of its carbohydrate-binding activity, promoting survival of embryonic sensory neurons and B lymphocyte maturation. The concept of moonlighting proteins has not been applied to microbial pathogens, agents that mimic many actions of their hosts in their quest for survival in the hostile environment. We find that, like PGI, the *T. cruzi* trans-sialidase moonlights as a parasite-derived mimic of neurotrophic factors (PDNF), as it binds and activates the nerve growth factor receptor TrkA to promote neurite extension and survival of neurons and glial cells, whether they are infected with *T. cruzi* or not. PDNF actions are independent of its carbohydrate-binding actions; which can be reproduced by PDNF-based synthetic peptides. The trans-sialidase/PDNF could play a role in *T. cruzi* survival in the nervous system and be a useful for neurodegenerative diseases such as Parkinson's disease.

L117
ANALYSIS OF *TRANS*-SIALIDASE ACTIVITY

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Trans-sialidase (E.C. 3.2.1.18) catalyses the transfer of preferably α2,3-linked sialic acid to another glycan or glycoconjugate, forming a new α2,3-linkage to galactose or *N*-acetylgalactosamine. Because of its great relevance to the pathogenicity of trypanosomes and its possible applications in biotechnology, interest in this enzyme increased rapidly within the last decade. To facilitate research on *trans*-sialidase, we developed a non-radioactive screening test for monitoring *trans*-sialidase activity (1).
In this assay, 4-methylumbelliferyl-β-D-galactoside (MUGal) is used as acceptor substrate and sialyllactose as donor to fluorimeterically detect enzyme activity in the low mU range. The test consists of two steps, first, the incubation of *trans*-sialidase with the substrates in closable 96-well-plates and secondly, the separation of the formed product 4-methylumbelliferyl-β-D-sialylgalactoside (MUGalNeu5Ac) from the acceptor MUGal using anion exchange chromatography in 96-filter-well-plates following acid hydrolysis of MUGalNeu5Ac and detection of released methylumbelliferone (MU).
The test can be applied to screen a large number of samples quickly, sensitively and reliably e.g. after chromatography runs during enzyme purification and for monitoring *trans*-sialidase activity during the production of monoclonal antibodies (2). Additionally, it can be used for testing substrates and inhibitors and for applying kinetic studies. This assay reveals to be advantageous compared to other tests using radioactive or chromogenic substrates.

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L118
TRANS-SIALIDASE FOR CONVENIENT SYNTHESES OF COMPLEX OLIGOSACCHARIDES

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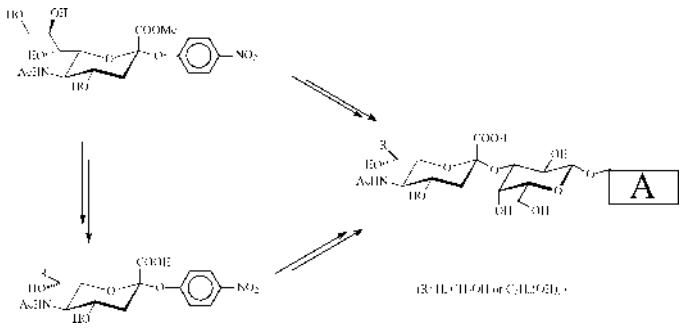
In Chagas disease trans-sialidase from *Trypanosoma cruzi* causes the transfer of Neu5Ac from a human host cell to the cell epitope of the pathogen. This unusual transfer mechanism enables the pathogen to protect its own cell surface against recognition by the human immune system. Interestingly, this enzyme belongs to the superfamily of the sialidases but shows merely transferase activity in the presence of a suitable acceptor molecule. Thus, trans-sialidase enables the transglycosylation of natural and several non natural donor substrates such as *p*NP-Neu5Ac to Galβ-R derivatives leading to a large variety of complex and biologically active oligosaccharides. Further, non naturally occuring oligosaccharides could be obtained, and subsequently used as building blocks for convenient syntheses of more complex glycoconjugates in very good yields.
The distinct transferase activity and the high acceptor specifity allows efficient approaches to complex oligosaccharides such as Neu5Ac-α(2-3)Gal-β(1-4)GlcNHAc-αAll in a tandem one pot synthesis. In a first step GlcNHAc-αAll was glycosylated with *p*NP-Gal employing β-galactosidase from *Bacillus circulans*. Subsequently the disaccharide was *in situ* transsialylated with *p*NP-Neu5Ac and trans-sialidase from *Trypanosoma cruzi*. After a fast purification by RPC the trisaccharide was obtained in acceptable yields and quantities.



Further, potential donor substrates were synthesized with modifications of the glycerol chains by single or double periodate cleavage of the *p*NP-Neu5Ac glycoside followed by reduction of the corresponding carbonyl compounds with cyanoborohydrate. These novel Neu5Ac mimetics could be obtained in excellent yields. Surprisingly, these octunolosonic and heptulosonic acid derivatives were recognized by trans-sialidase and transglycosylated in comparable yields with lactose derivatives as acceptor substrates, to accomplish a tandem one pot synthesis towards novel LacNAc glycoside analogues.

L119
STRUCTURAL AND KINETIC STUDIES INTO THE CATALYTIC MECHANISM OF *TRYPANOSOMA CRUZI* TRANS-SIALIDASE

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Trans-sialidases are GPI-anchored surface proteins expressed during the developmental stages of some trypanosomal parasites such as *Trypanosoma cruzi* and *T. brucei*. Trans-sialidases (CAZy family GH 33) belong to the super family of sialidases (GH 33, 34, 83), but rather than catalysing the hydrolysis of sialic acid, they preferentially transfer α-(2,3)-linked sialic acid to terminal galactose residues on the surface of these parasites with an overall retention of anomeric configuration. Sialidases and trans-sialidases are expressed by a number of pathogens and are seen as potential targets for the chemotherapeutic treatment of various diseases. However, many fundamental questions about the mechanism remained, especially concerning the identity, or even existence, of a catalytic nucleophile.
This lecture describes the synthesis and use of mechanism based inactivators, combined with mass spectroscopy, kinetic studies and protein crystallography, to confirm that *T. cruzi* trans-sialidase operates through a catalytic mechanism involving the transient formation of a covalent glycosyl-enzyme intermediate to a tyrosine residue. This is the first case in which tyrosine has been unequivocally shown to play such a role in the catalytic machinery of a retaining glycoside hydrolase, and a rationale for this choice of nucleophile will be presented. We have also obtained X-ray crystal structures of *T. cruzi* trans-sialidase bound to several substrate analogues and used these structures to map conformational changes in the substrate along the reaction coordinate. Finally, our current efforts towards the design and synthesis of new mechanism-based inactivators of sialidases as potential therapeutics will also be discussed.

L120
STUDIES ON THE SIALYLTRANSFERASE THAT FORMS THE NeuAcα2-6GlcNAc STRUCTURE IN OLIGOSACCHARIDE

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Rat, Human and bovine glycoproteins contain the structure NeuAcα2-6 GlcNAc. The sialyltransferase that adds sialic acid is described as ST6GlcNAcI and it has a specificity for addition to NeuAcα 2-3Galβ1-3GlcNAc terminal trisaccharides of oligosaccharides of the N- and O-type. We have used oligosaccharides of defined structure to act as acceptors for this enzyme, and more recently we have developed macromolecular acceptors for the enzyme. Starting materials were fetuin and bovine α-1 acid glycoprotein. Fetuin contains O- and N- linked chains. To remove O- linked chains fetuin was treated with sodium borohydride in presence of acetate/EDTA which protects N-linked chains while specifically cleaving the O-linked chains. NeuAc was then removed with neuraminidase or dilute acid treatment and Galβ 1-4 was removed by Streptoccus pneumoniae galactosidase. The Galβ 1-3 chains that remained were then recharged with sialic acid using ST3GalII to produce the correct acceptor structure. At each step acceptors were checked for their ability to react with ST6GalI,ST3GalIII and ST3GalII. Bovine α-1 acid glycoprotein does not contain O-linked chains so borohydrate treatment was not necessary. The same approach that was used for fetuin was used with this protein to prepare acceptors. Both acceptors were used in kinetic studies with rat liver Golgi as source of enzyme, at each stage checking that the correct product of the reaction was formed. Using rats suffering from the Acute Phase response it was found that ST6GlcNAc I was an acute phase reactant.
Supported by NSERC, Canada.

L121
MOLECULAR CHARACTERIZATION OF THE POLYSIALIC ACID SPECIFIC O-ACETYLTRANSFERASE OF *ESCHERICHIA COLI* K1

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Escherichia coli K1 (*E. coli* K1) causes sepsis and meningitis in neonates. The capsule composed of poly-alpha-2,8-linked sialic acid (polysialic acid; polySia) is the major virulence factor, essential for serum resistance and vital passage of the blood brain barrier. Several strains modify their capsular sialic acids by O-acetylation at C-7 and C-9 position. However, little is known on the role of capsule O-acetylation during infection of *E. coli* K1 and the polySia specific O-acetyltransferase.
By homology search of the partially sequenced genome of the *E. coli* K1 strain RS218, we identified a potential O-acetyltransferase gene (designated *oat*K1) which was part of the 40 kb genome of a K1 specific prophage. After recombinant expression in *E. coli* BL21(DE3) we succeeded in affinity purification of an active enzyme catalyzing the transfer of acetyl groups from acetyl-coenzyme A to polySia. Using computer-assisted modelling, we predicted a 3D-structure composed of a central left-handed parallel beta-helix (LβH) fold with one protruding loop which assembles into a homo-trimer. Two amino acids highly conserved in a subset of the LβH-family of acyltransferases were identified and subsequent amino acid exchanges to alanine abrogated the enzymatic activity *in vitro* and *in vivo*. Our results indicate high structural similarity of OatK1 to other members of the LβH-family, although structurally diverse acceptor substrates are used by these enzymes.

L122
CONFORMATION AND ORIENTATION OF SPHINGOLIPIDS IN LIPID MONOLAYERS.

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Lipid monolayers at the air-water interface serve as model systems for biomembranes or lipid bilayers, since a monolayer represents half of a membrane. Infrared-reflection-absorption-spectroscopy (IRRAS) is ideally suited to study the conformational behavior and orientation of lipids in monolayers. The angular dependence of the IR-spectra provides additional information about the orientation of certain moieties in the molecules, for instance, the alkyl chains or carbonyl groups.
We performed a systematic study of the conformational and orientational behavior of the gangliosides GM1, GM2, and GM3 as pure lipid monolayers and in mixtures with phospholipids, such as dipalmitoyl-phosphatidylcholine (DPPC). The study was supplemented by epifluorescence microscopy obtaining information on the domain formation in the pure and mixed monolayer systems. A comparison was made with the behavior of the building blocks of the complex gangliosides, namely the ceramide and a glycosylceramide. Finally, also sphingomyelin, a sphingolipid with a phosphocholine headgroup was investigated.
GM3 films show a transition to a liquid-condensed phase at higher surface pressure, whereas the gangliosides GM1 and GM2 show only liquid-expanded phases. A systematic change in tilt angle of the chains as a function of the ganglioside headgroup size was observed. In mixed films we found evidence for fluid-fluid phase separation and also for an induction of a liquid-condensed phase at lower surface pressure. Data obtained on lipid monolayers are important for the interpretation of the behavior of bilayers of the same systems.

L123
FUNCTIONAL EXPRESSION OF THE CMP-SIALIC ACID TRANSPORTER IN *ESCHERICHIA COLI*.

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Introduction:
Transport of nucleotide sugars into the Golgi is exclusively mediated *via* nucleotide-sugar transporters (NST). Defining the transport characteristics of recombinant NST has become a major challenge since true zero background systems are widely absent; alternative assay systems are clearly needed. Using the mouse CMP-sialic acid transporter (CST) as a model system, we investigated expression of functional CST in bacteria.
Experimental Approaches:
CST was expressed, with and without the OmpA leader peptide, in *E. coli* under the control of the inducible *trp/lac* promoter.
CST (without OmpA) was solubilised from inclusion bodies with urea and renatured through stepwise removal of the denaturant. Transport activity was determined following reconstitution of renatured CST into proteoliposomes.
CST (with OmpA) was expressed in the membrane fraction of *E. coli*. Whole cell assays were performed by incubating CST-OmpA-expressing *E. coli* cells (± spheroplasting) with CMP-[^{3}H]Neu5Ac. *E. coli* cells were subsequently collected on

Multiscreen plates and the incorporated radioactivity determined.
Results:
CST expressed without the OmpA leader peptide was quantitatively sequestered in the *E. coli* inclusion body pellet. However, following solubilisation, renaturation and reconstitution into proteoliposomes, CST was able to specifically transport CMP-Neu5Ac.
When expressed as a CST-OmpA fusion protein, CST was localised to the periplasmic membrane. Preliminary transport assays indicated that speroplasted *E. coli* cells expressing CST were able to transport CMP-[^{3}H]Neu5Ac.
Conclusions:
This study illustrates the viability of NST expression in bacteria, thus providing an alternative heterologous expression system for characterising putative NSTs, as well as adequate quantities of functional protein for downstream evaluation.

L124
TRANSCRIPTIONAL REGULATION OF GLYCOSYLTRANSFERASE GENES

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Glycolipid (GSL) biosynthesis is strictly regulated by the activities of glycosyltransferases (GTs) and is necessarily controlled at the levels of gene transcription and posttranslational modification. Cells can switch between expressing simple and complex GSLs or between different series of GSLs during differentiation. Although post-translational modifications of GTs as well as the compartmentation of GTs and substrates play important regulatory roles in GSL biosynthesis, the expression of GTs themselves are tightly regulated at the transcription levels. To date, the promoters of a number of GT genes for GSL synthesis have been sequenced and analyzed, and this information is useful in defining the control mechanisms of GT expression. The common feature of these promoters is that all of them are TATA-less and contain no CCAAT box, characteristic of many house-keeping genes. Thus, the transcription of GT genes requires additional factors for regulation. It is interesting that these GTs contain GC-rich sequences in the proximal promoter regions, which have multiple transcription factor-binding sites. For example, the mouse GM3-synthase gene contains a 5'-flanking 254-bp DNA fragment with two Sp1- and six AP2-binding sites that are essential for enhancing the basal activity of the promotor in mouse Neuro-2a cells. In addition to the Sp1 and AP2 sites, human UDP-galactose: ceramide galactosyltransferase (hCGT) promoter contains a GC-box (-267/-259) and a CRE (-697/-690) site that are critical. Evidence will be provided that these elements function cooperatively and CRE is essential for regulating the cell-specific expression of the hCGT gene.

L125
UNDERSTANDING THE ORGANIZATION OF GLYCOLIPID SYNTHESIS IN THE GOLGI COMPLEX

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Glycolipid glycosyltransferases are synthesized in the endoplasmic reticulum (ER) and transported towards their site of residence, the Golgi complex. They form different physical associations which overlap along the proximo-distal axis of the Golgi, although those involved in the synthesis of simple glycolipid species concentrate in the proximal-while those for complex species in the distal- (trans Golgi and trans Golgi network or TGN) aspects of the organelle. The N-terminal domain [comprising the **c**ytoplasmic **t**ail (ct), the **t**rans**m**embrane **r**egion (tmr) and few amino acids of the stem region] bears information for the journey from the ER to the Golgi and for the formation of associations as do the full length forms of these enzymes. Selective concentration at ER exiting sites depends on interactions of a [RK](X)[RK] motif in the ct with the small GTPase Sar1 involved in COPII vesicle formation, while complex formation seems to depend on interactions among the tmrs. Currently we are investigating whether the N-terminal domain of SialT2, localized in the proximal Golgi, and the one of GalNAcT, a distal Golgi enzyme, are able to concentrate reporter proteins in the corresponding sub-Golgi compartment. Double color fluorescence microscopy in single CHO-K1 cells, and sub-cellular fractionation showed that SialT2 N-terminal domains concentrate spectral variants of the GFP in a proximal- and that of GalNAcT in a distal- sub-Golgi compartment. Experiments exchanging the cytoplasmic tails of SialT2 and GalNAcT indicate that information for proximal or distal Golgi localization is associated to the ct of these N-terminal domains.

L126
GLYCOSPHINGOLIPID STRUCTURE AND MEMBRANE MICRODOMAINS AS DETERMINANTS OF CAVEOLAR ENDOCYTOSIS.

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Caveolae are a subset of plasma membrane (PM) microdomains that are enriched in glycoshingolipids (GSLs) and cholesterol, and are associated with the protein, caveolin-1. These structures appear as flask-shaped invaginations at the PM and serve various functions including organization of some signaling machinery, and transcytosis and potocytosis in specialized cell types. Caveolae are also involved in the clathrin-independent endocytosis of certain membrane

components (e.g., GSLs; β1-integrins) as well as some bacteria, viruses, and toxins. We have previously shown that fluorescently labeled lactosylceramide (BODIPY-LacCer) and other GSL analogs are internalized almost exclusively *via* caveolae in human skin fibroblasts and other cell types, however the molecular basis for this selectivity is not known. In this lecture, evidence for the caveolar uptake of these lipid analogs and our studies demonstrating the stimulation of caveolar endocytosis by exogenous GSLs or cholesterol will first be reviewed. Recent work showing the importance of GSL structure on caveolar endocytosis will then be presented. Through systematic modification of the GSL head group, fatty acid, and sphingosine moieties, we found that the stereochemistry of the sphingosine base was critical for GSL uptake *via* caveolae. Surprisingly, a non-natural GSL stereoisomer inhibited the caveolar internalization of other markers endocytosed by this mechanism. Finally, we show the formation of GSL microdomains at the PM of living cells could be detected by monitoring the monomer-excimer fluorescence of the BODIPY-analogs. These microdomains correlated with the ability of the GSL stereoisomers to be internalized *via* caveolae. Supported by USPHS Grant GM22942.

L127
REGULATION OF SPHINGOLIPID AND GLYCOPROTEIN TRANSPORT IN APICAL MEMBRANE BIOGENESIS IN HEPG2 CELLS

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In polarized HepG2 cells we have identified an endosmal organelle, SAC, which fulfills a prominent role in the biogenesis of the canalicular membrane by means of its ability to sort and redistribute apical and basolateral sphingolipids. Intrinsic activation of protein kinase A (PKA) appears instrumental in this process. Oncostatin M (OSM), an IL-6 type cytokine activates membrane polarity development, upstream of PKA activation. This requires recruitment of the gp130 receptor into detergent resistant membrane microdomains at the basolateral surface, thereby activating SM transport from SAC to the apical membrane. This mechanism couples the biogenesis of a plasma membrane domain to the regulation of intracellular transport in response to an extracellular stimulus.
The OSM and the PKA-dependent pathway appears to be carefully regulated by intracellular levels of free sphinganine, and dihydroceramide synthase, the predominant enzyme responsible for sphinganine turnover, is a target for cell polarity stimulating cAMP/PKA signaling cascades: a significant reduction in sphinganine levels is seen in cAMP/PKA-stimulated cells. A potential functional link is suggested between sphinganine turnover and $p27^{Kip1}$-mediated cell cycle control in regulating plasma membrane polarity development.
OSM stimulated the association of PKA-RIIα with the centrosome/Golgi region of hepatocytes. The evidence indicates that this anchoring is required for the efficient and coordinated apical delivery along the biosynthetic pathway of a subset of newly-synthesized apical (glyco)proteins, including MDR1/P-glycoprotein and distinct de novo synthesized glycosphingolipids.
Specifically, glucosylceramide synthesis is implicated as an important parameter for efficient Golig-to-apical surface transport and apical retention of MDR1 in this pathway.

L128
FUNCTIONAL IMPLICATION OF INTRACELLULAR LOCALIZATION OF GANGLIOSIDE GD3 IN MALIGNANT MELANOMA CELLS

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Introduction: Although ganglioside GD3 has been considered to be a human melanoma-associated antigen, its biological role has not been well understood. Therefore, we studied the implication of GD3 in the malignant properties of melanoma cells with special focus on the effects on cellular signals.
Approaches: A GD3-lacking mutant line, SK-MEL-28N1 was transfected with GD3 synthase cDNA to establish GD3-positive cell lines. Involvement of various signaling molecules in GD3-induced changes was analyzed with specific inhibitors, such as U0126 (ERK1/2), wortmannin (PI3-kinase), and siRNAs (p130Cas, paxillin). Methyl-b-cyclodextrin (MBCD) was used for lipid raft disruption.
Results: GD3 expression in the mutant melanoma line resulted in the restoration of cell proliferation and invasion activities. FCS treatment of cells resulted in enhanced tyrosine phosphrylation of p130Cas and paxillin in GD3-expressing cells. Involvement of these adaptor molecules in cell growth and invasion was verified with knock down using siRNAs. Moreover, MBCD treatment reduced tyrosine phosphorylation of these adaptors, suggesting that the enhanced signaling with GD3 was mediated with lipid rafts. Then, intracellular distribution of GD3 was analyzed, showing its localization at leading edges together with p130Cas and paxillin. While GD3 was mainly detected in lipid rafts in a melanoma cell line, it was widely distributed inside/outside of rafts in Caveolin-1-overexpressing cells. This change might have caused reduced

efficiency of GD3-mediated signals and suppressed cell growth.
Conclusions: These results suggested that expression levels and distribution patterns of GD3 are crucial to determine the quality/quantity of cell signals.

L129
THE ROLE OF GOLGI α-MANNOSIDASE II IN THE BIOSYNTHESIS OF PLANT COMPLEX N-GLYCANS

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Golgi α-mannosidase II (GMII) is a key enzyme in the conversion of hybrid to complex type N-linked oligosaccharides. Its central role in N-glycan biosynthesis is well characterised in animals. However, plant orthologues of the enzyme have not yet been identified.
In this study, we cloned and molecularly characterised GMII from *A. thaliana* (AtGMII). The amino acid sequence deduced from the cDNA reveals a type II membrane protein topology. A recombinant form of the enzyme expressed in insect cells hydrolyses p-nitrophenyl α-D-mannopyranoside and is inhibited by swainsonine. A detailed analysis of the substrate specificity revealed efficient processing of the physiological GMII substrate $GlcNAcMan_5GlcNAc_2$ to $GlcNAcMan_3GlcNAc_2$. AtGMII also converted $GlcNAcMan_5XylGlcNAc_2$ into $GlcNAcMan_3XylGlcNAc_2$, while $Man_5GlcNAc_2$ was not hydrolysed. N-glycan analysis of AtGMII knock-out plants using MALDI-TOF-MS revealed the presence of predominantly hybrid N-glycans carrying β1,2-xylose and core α1,3-fucose residues. Our results provide evidence that AtGMII plays a central role in the formation of plant complex N-glycans. Furthermore, we show that at least two alternative pathways leading to complex N-glycans may occur in plants.
By transient expression of truncated AtGMII constructs fused to GFP, we demonstrate that the transmembrane domain and ten amino acids from the cytoplasmic tail are sufficient for retention in the Golgi apparatus. A GFP fusion containing only the transmembrane domain of AtGMII was predominantly retained in the ER, a result which indicates the presence of a motif promoting ER export within the ten amino acids of the cytoplasmic tail.

L130
BIOSYNTHESIS AND SUBCELLULAR LOCALIZATION OF DEAMINONEURAMINIC ACID (KDN) IN MAMMALIAN CELLS

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Sialic acids (Sia) are a family of nine carbon, 2-keto-3-deoxy sugars found in viruses, bacteria, and higher animals. KDN (deaminoneuraminic acid, 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid) constitutes a unique group of Sia family because it possesses distinct properties, such as insensitivity to various sialidases, the presence at the terminal position of polysialic acid as a stop signal for the elongation, and the higher levels of expression in certain cancer cells. We previously demonstrated that, in mammalian cells, KDN is usually expressed at very low levels, and that the level of free KDN increases when the cells were cultured in mannose-rich media.
Our objective is to understand the regulatory mechanism of KDN expression in mammalian cells. In this study, we examined effects of suppression of Neu5Ac 9-phosphate synthase (NPS) activity by siRNA for NPS on the biosynthesis of KDN. We showed that the suppression of the NPS activity resulted in inhibition of the mannose-induced increase of KDN in B16 cells. We also show that the biosynthesis of KDN was inhibited by addition of *N*-acetylmannosamine, a precursor sugar of Neu5Ac. These results suggest that NPS is involved in the synthesis of KDN in mammalian cells. Furthermore, we analyzed the amount of KDN in subcellular fractions of mouse cultured cell lines, and demonstrated that KDN showed different intracellular localization from Neu5Ac. These results suggest that KDN and Neu5Ac share the common metabolism, but take different intracellular sorting in mammalian cells.

L131
CLONING AND EXPRESSION OF MOUSE CYTOSOLIC ALPHA-D-MANNOSIDASE

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Alpha-D-mannosidase activity is involved in both biosynthesis and catabolism of N-linked and free oligomannosides.The neutral/cytosolic alpha-D-mannosidase can cleave alpha 1,2, alpha 1,3, and alpha 1,6 linked mannose residues, is stimulated by cobalt, and is inhibited by furanose analogues swainsonine (SW) and 1,4-dideoxy-1,4-imino-D-mannitol (DIM). The enzyme is involved in the degradation of oligomannosides derived from dolichol intermediates and from the degradation of newly synthesized glycoproteins. It was demonstrated an immunological relationship between the rat endoplasmic reticulum alpha-D-mannosidase and the cytosolic alpha-D-mannosidase. A cDNA encoding the mouse cytosolic alpha-D-mannosidase was obtained by RZPD, Berlin, Germany. Comparison of the mouse genomic sequence

with the cDNA sequence revealed the presence of 25 introns within the cytosolic alpha-D-mannosidase gene. The gene spans 11.5 kb from the major transcription initiation site to the RNA cleavage site. The transcription initiation site of mouse cytosolic alpha-D-mannosidase was mapped 170 bases upstream of ATG codon using 5' RACE . Northern blotting analysis revealed differential expression of a major transcript of 3.8 kb in all tissues examined. COS cells transfected with the cDNA showed a 20-fold increase of cytosolic alpha-D-mannosidase activity. This enzyme activity is stimulated by cobalt and inhibited by DIM. Furthermore the specificity of the expressed activity was demonstrated using the radiolabeled substrates Man9GlcNAc1 and Man9GlcNAc2. In fact the first one (Man9GlcNAc1) was hydrolized by the expressed enzyme to Man5GlcNAc, whereas the second one (Man9GlcNAc2) was not cleaved. These properties confirms that the cloned cDNA encodes the cytosolic alpha-D-mannosidase.

L132
DIHYDROCERAMIDE:SPHINGANINE C4 HYDROXLATION FOR PHYTOGLYCOLIPID BIOSYNTHESIS REQUIRES DES2 HYDROXYLASE AND CYTOCHROME b5

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Phytoglycolipids distribute in a unique manner in our body and are enriched in the microvillous membrane of mammalian small intestine and the apical membrane of kidney tubule epithelial cells. This feature is conserved in mammals. DES2 (degenerative spermatocyte 2) has been identified as a bi-functional enzyme to produce phytoceramide and ceramide from dihydroceramide. Molecular mechanism involved in the C-4 hydroxylation has not been studied in detail. We report that the C-4 hydroxylation requires an electron transfer system including cytochrome b5, and the hydroxylase activity is reconstituted in an *in vitro* assay with a purified recombinant DES-2. Mouse FLAG-tagged DES2 was expressed in Sf9 cells and purified by solubilization with digitonin and anti-FLAG antibody affinity column chromatography. The activity of dihydroceramide:sphinganine C-4 hydroxyalse was reconstituted with the purified FLAG-DES2, the membrane form of cytochrome b5 (mb5), and bovine erythrocyte membrane. Interestingly, mb5 was not replaced with the soluble form of cytochrome b5. These lines of evidence indicate that the hydroxylation requires a complex formation with DES2 and mb5 through their membrane-spanning domains.

L133
GLYCOPHENOTYPE OF CYSTIC FIBROSIS (CF) AIRWAY CELLS: A ROLE FOR WTCFTR IN GOLGI ORGANIZATION

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In CF, a common lethal genetic disease, patients lack functional copies of wtCFTR, an apical chloride channel of epithelial cells. A glycophenotype of decreased NeuAc and increased Fuc characterizes the surface membranes of CF airway epithelial cells. This glycophenotype is reversed when wtCFTR is present (1).
Several approaches were used to test our hypothesis that the altered glycosylation in CF results from a mislocalization of the glycosyltransferases involved in terminal glycosylation (2). The most compelling data was found after sucrose density gradient centrifugation and separation of the Golgi region as marked by proteins GS27 and p230. The activities of NeuAcT, FucT and GalT were only partially located within the Golgi area of the gradients from CF cells. However, when wtCFTR was present in the cells the transferase activities were found within the Golgi. Since CFTR is an ion channel, the effect of a weak base on the location of the glycosyltransferases within the Golgi was examined. Comparing GalT activity in the Golgi from alkali treated and non-treated CF cells it was found that the alkali treated cells had a return of GalT to Golgi localization.
Additionally, confocal microscopy of NeuAcT showed a diffuse pattern surrounding the nucleus in the CF cells compared to discrete Golgi localization seen in the non CF cells. When the proteins of the COG and COP complexes were analyzed by confocal microscopy, two proteins, COG 8 and BCOP, had a similar diffuse pattern in the CF cells. The conserved oligomeric Golgi (COG) complex proteins are involved in proper localization of Golgi proteins (3). This also supports our hypothesis that CFTR plays a role in the organization of proteins in the Golgi.
Supported in part by NIH Medical Student Research Fellowship (AR), RWJMS-UMDNJ and the Nurmi Foundation.
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L134
A CLASS OF POLYENOIC FUCOSYLATED GLYCOSPHINGOLIPIDS IN GERM CELLS IS ESSENTIAL FOR SPERMATOGENESIS

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Mice require complex glycosphingolipids (GSLs) for proper spermatogenesis. Detailed analysis of mice deficient in specific GSLs revealed that ganglio-series GSLs are expressed in germ and non-germ cells and can be associated with male fertility. In contrast to non-germ cells that express the classical "brain-type" gangliosides (GM1a, GD1a, GD1b, GT1b and GQ1b) with saturated long chain fatty acids, germ cells express a novel class of fucosylated GSLs (FGSLs) [IV^2FucGA1, IV^3Galα(IV^2Fuc)GA1, IV^3GalNAcα(IV^2Fuc)GA1 and (IV^3GalNAcβ3Galα)(IV^2Fuc)GA1 and the corresponding 4 GM1-derivatives] that is caracterized by the predominant incorporation of highly unsaturated (4-6 double bonds) very long chain fatty acid moieties (C26-C32). Histological analysis reveals differential expression of the individual members of this class in germ cells. The highly unsaturated very long chain fatty acids provide these FGSLs with different physico-chemical membrane properties compared to classical GSLs; this might lead to specific functions for FGSLs in germ cells. From comparison of fertile and infertile mouse mutants we conclude that polyenoic FGSLs of germ cells are essential for spermatogenesis. We hypothesize that highly unsaturated FGSLs stabilize Sertoli cell-germ cell interactions, which are important for germ cell differentiation.

L135
MOUSE BRAIN Na/K-ATPASE β1-SUBUNIT HAS A K-DEPENDENT CELL ADHESION ACTIVITY FOR β-GlcNAc-TERMINATING GLYCANS

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Mammalian brain glycoproteins contain unique β-N-acetylglucosamine (GlcNAc)-terminating N-glycans. When mouse embryonic neural cells were cultured in dishes with different concentrations of *Psathyrella velutina* lectin (PVL) that binds to β-GlcNAc-terminating glycans, the cell proliferation and neurite extension were inhibited in a dose-dependent manner. Immunoblot analysis showed that the phosphorylation of mitogen-activated protein kinases and protein kinase C are inhibited by culturing the cells in PVL-coated dishes but are restored by the addition of the haptenic sugar into the medium, indicating that β-GlcNAc-terminating N-glycans are important for neural cell growth. To examine whether or not neural tissues contain a natural receptor for β-GlcNAc-terminating N-glycans, solubilized membrane proteins from mouse brain were applied to a GlcNAc-Agarose column, and a 48 KDa protein was obtained as a major GlcNAc-binding protein. The N-terminal amino acid residues showed the protein to be a mouse Na/K-ATPase β1-subunit. When the recombinant FLAG-β1-subunit expressed in Sf-9 cells was applied to a GlcNAc-Agarose column, only glycosylated 38- and 40-KDa proteins bound to the column. In the absence of KCl, the proteins did not bind to a GlcNAc-Agarose column but bound in the presence of KCl at the concentration above 1 mM. Inclusion of anti-β1-subunit antibody or chitobiose in cell aggregation assays using mouse neural cells resulted in inhibition of cell aggregation. These results indicate that the Na/K-ATPase β1-subunit is a new type of lectin that binds to β-GlcNAc-terminating N-glycans, and may be involved in neural cell interactions.

L136
STRUCTURAL CHARACTERIZATION AND QUANTIFICATION OF SHIGA TOXIN 1-BINDING Gb3/CD77 GLYCOSPHINGOLIPIDS ON HUMAN BRAIN MICROVASCULAR ENDOTHELIAL AND EA.hy 926 CELLS

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Shiga toxin 1 (Stx1)-producing enterohemorrhagic *Escherichia coli* strains of serotype O157:H7 cause

hemorrhagic colitis, which is often followed by hemolytic-uremic syndrome (HUS) and/or acute encephalopathy. Human brain microvascular endothelial cells (HBMEC) and EA.hy 926 cells are known to exhibit low and high Stx1 susceptibility, respectively. The neutral glycosphingolipid (GSL) globotriaosylceramide (Gb3/CD77) acts besides globotetraosylceramide (Gb4) as the preferential receptor for Stx1. The aim of this study was to structurally characterize the neutral GSLs and to quantify the expression of Gb3/CD77 in HBMEC and EA.hy 926 cells.
Endothelial cells were propagated on Cytodex 3 microcarriers. Extracted GSLs were separated by thin-layer chromatography (TLC) and immunostained on the TLC plate. Anti-Gb3 and anti-Gb4 positive bands were extracted and the GSLs were structurally characterized by nanoESI-QTOF tandem mass spectrometry. Quantification was performed by ELISA and TLC immunostaining.
Neutral GSLs of the globo-series were the dominant GSLs in both cell lines. HBMEC exhibited an almost equal ratio of Gb3 and Gb4 (42% vs. 37%). EA.hy 926 cells showed an extreme increase of Gb3 and almost complete absence of Gb4 (less than 0.1%). MS analysis demonstrated for both cell lines the presence of Gb3(d18:1) species containing C24:0, C24:1, C22:0, and C16:0 fatty acids. The calculation of Gb3/CD77 molecules revealed an average number of 2.1 x 10^8 Gb3 molecules per HBMEC and 6.4 x 10^{10} Gb3 molecules per EA.hy 926 cell. Thus, this 300-fold increase correlates with the enhanced susceptibility of EA.hy 926 cells and renders this cell line an excellent target for Gb3/CD77-binding shiga toxins.

L137
SIGLEC-14: A NOVEL SIALIC ACID RECEPTOR UNDERGOING CONCERTED EVOLUTION WITH SIGLEC-5 VIA GENE CONVERSION IN MULTIPLE PRIMATE SPECIES

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Siglecs are vertebrate lectins that belong to the immunoglobulin superfamily, and recognize sialylated glycans. A CD33-related subfamily is expressed on immune cells, each typically containing a cytosolic immunoreceptor tyrosine-based inhibitory motif (ITIM), assumed to mediate negative regulatory signaling.
Human Siglec-5/CD170 has four extracellular immunoglobulin-like domains and a cytosolic ITIM, and is encoded by the *SIGLEC5* gene on chromosome 19. Investigation of a potential gene adjacent to *SIGLEC5* resulted in cloning of a novel molecule designated Siglec-14, which has three immunoglobulin-like domains. The first two domains only showed almost complete sequence identity with those of Siglec-5, indicating gene conversion between *SIGLEC5* and *SIGLEC14.* Remarkably, *SIGLEC5* and *SIGLEC14* in other primates (chimpanzee, gorilla, orangutan and baboon) also showed evidence for similar independent gene conversions. Moreover, the "essential arginine" critical for sialic acid recognition in both Siglec-5 and Siglec-14 is missing in most great apes. Siglec-5 and Siglec-14 thus appear to be paired receptors, undergoing concerted evolution via gene conversion, and presumably working in coordination to regulate immune responses.
The cytosolic tail of Siglec-14 lacks a typical ITIM. However, its transmembrane domain contains a positively charged residue, suggesting a different signaling mechanism from that of Siglec-5. Siglec-14 transcripts were found in hematopoietic tissues such as bone marrow, showing a similar distribution to Siglec-5. Notably, most available anti-Siglec-5/CD170 antibodies cross-react with Siglec-14. Thus, some prior reports of the expression pattern of Siglec-5 in humans may actually represent Siglec-14 expression.

L138
DEMONSTRATION OF *N*-GLYCAN-BINDING ACTIVITY OF HSP70 AND ITS INPORTANCE IN THE CHAPERONING ACTIVITY

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We previously identified the sea urchin egg 350-kDa sperm-binding protein (SBP) as a sialic acid-binding lectin [Maehashi *et al*. (2003) *J. Biol. Chem*. **278**, 42050]. Interestingly, SBP contains highly homologous amino acid sequences to heat shock protein (Hsp) 70 family. We thus suspected that Hsp70 family has the carbohydrate-binding ability. In this study, based on the following experiments, we demonstrate that cytosolic Hsp70 has the *N*-glycan-binding activity and suggest that this property may be important in quality control and ER-associated degradation of glycoproteins. (i) The recombinant Hsp70 was shown to bind ovalbumin (OVA), when the binding was assessed by an ELISA-based assay. Of OVAs of different glycan types, Hsp70 preferred OVA of a high mannose-type to OVA of a hybrid-type. In addition, the Hsp70-OVA binding was inhibited by trimannose (Manα1,3(Man1,6)Man) and glycoasparagine ($Man_6GlcNAc_2Asn$). (ii) To determine the carbohydrate-binding domain of Hsp70, a pull-down assay of recombinant proteins of Hsp70 with various deleted domains by the RNase B-Sepharose was performed. The ATPase domain of Hsp70 was shown to bind the RNase B-Sepharose, and the binding was released by trimannose, thus suggesting that the ATPase domain is responsible for the *N*-glycan-

binding activity. (iii) To assess the function of the *N*-glycan-binding activity of Hsp70, the chaperoning activity of Hsp70 was measured by the aggregation-inhibition assay. The aggregation of the chemically denatured OVA was inhibited in the presence of Hsp70/HDJ-1 complex, and this aggregation inhibition was inhibited by trimannose. These results indicate the importance of the *N*-glycan-binding activity for the chaperoning activity of Hsp70.

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L139
SPECIFIC LABELING OF CYTOPLASMIC PNGASE BY OLIGOSACCHARIDE-RELATED COMPOUNDS

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Peptide:*N*-glycanase (PNGase) is the deglycosylating enzyme which removes *N*-linked glycan chains from *N*-glycopeptide/glycoproteins (1). Recent studies have revealed that the cytoplasmic PNGase is involved in the degradation of misfolded/unassembled glycoproteins (2). This enzyme has a catalytic triad of Cys, His, and Asp for its enzymatic activity (3), and also the protein is found to have high affinity to "free" *N*-linked glycan, that could potentially serve as a feed back inhibition mechanism of this enzyme (4). Previously the chemical synthesis of high-mannose type glycans and their derivatives were reported (5). Among them, the iodoacetamide-derivative (CHO-IAc) is an attractive candidate for efficient labeling/inhibitor of the cytoplasmic PNGase, because of its high-affinity towards free glycan as well as the occurrence of catalytic Cys. Indeed, we found that PNGase could be specifically-labeled with CHO-IAc. This compound was also found to be a strong inhibitor for the cytoplasmic PNGase. The mutant enzyme in which catalytic Cys was changed to Ala did not bind to CHO-IAc, suggesting that the catalytic Cys is the binding site for this compound. These results suggested that CHO-IAc could attack catalytic Cys for irreversible inhibition of the cytoplasmic PNGase. Possible use of CHO-IAc for PNGase-inhibitor will be discussed.

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L140
RECONSTITUTION *IN VITRO* OF THE GDP-FUCOSE BIOSYNTHETIC PATHWAYS OF *CAENORHABDITIS ELEGANS* AND *DROSOPHILA MELANOGASTER*

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Fucosylated glycans have a number of roles in disease and development and the genetic model organisms *Caenorhabditis elegans* and *Drosophila melanogaster* express novel types of oligosaccharides carrying fucose as well as some types which are conserved in comparison to mammals. The donor required by the fucosyltransferases synthesising these glycans is GDP-fucose, which is generated from GDP-mannose in three steps catalysed by two proteins. By homology we have identified and cloned cDNAs encoding these two proteins, GDP-mannose dehydratase (GMD; EC 4.2.1.47) and GDP-keto-6-deoxymannose 3,5-epimerase/4-reductase (GER or FX protein; EC 1.1.1.271), from both *Caenorhabditis* and *Drosophila*. Whereas the nematode has two genes encoding forms of GMD (*gmd-1* and *gmd-2*) and one GER-encoding gene (*ger-1*), the insect has, like mammalian species, only one homologue of each (*gmd* and *gmer*); as controls, we used one each of the two *Arabidopsis* homologues for both enzymes, *mur1 (gmd2)* and *ger1*. All corresponding cDNAs were separately expressed and the encoded proteins found to have the predicted activity as judged by HPLC assays; the product of the two *Drosophila* enzymes was also characterised by NMR. Identification of these genes opens up the possibility of manipulating the expression of all fucosylated glycans in these organisms.

L141
BIOSURFACTANTS OF MEL-A DRAMATICALLY INCREASE GENE TRANSFECTION VIA MEMBRANE FUSION

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Many microorganisms growing on water-insoluble substrates have been known to produce surface-active compounds called as biosurfactants. Although biosurfactants have been received increasing attention due to their special properties, there has been little information available until now of a role for them with regard to gene transfection. Thus, we studied here the effects of three different biosurfactants, mannosylerythritol lipid MEL-A, MEL-B, and MEL-C on gene transfection by cationic liposomes with a cationic cholesterol derivative. MEL-A consists of 4-*O*-(di-*O*-acetyl-di-*O*-alkanoyl-β-D-mannopyranosyl)-erythritol esterified two fatty acids and two acetic acids. MEL-B and MEL-C consist of 4-*O*-(mono-*O*-acetyl-di-*O*-alkanoyl-β-D-mannopyranosyl)-erythritol esterified two fatty acids and an acetic acids. The results showed clearly that a biosurfactant of mannosylerythritol lipid

A (MEL-A) dramatically increased the efficiency of gene transfection mediated by cationic liposomes with a cationic cholesterol derivative, but MEL-B and MEL-C did not. We also studied the localization of FITC-conjugated antisense DNAs in the target cells by fluorescence microscopy. The FITC-conjugated antisense oligonucleotides were temporarily on the plasma membrane of the target cells, thereafter they were transferred into the nucleus of the target cells. In the case of MEL-B and MEL-C, such localization of DNA was not observed both in the plasma membranes and in the nucleus. This demonstrates that MEL-A has great potential in the experiment of gene transfection and gene therapy mediated by non-viral vectors such as cationic liposomes.

Biochem. Biophys. Res. Commun., 289, 57 (2001).
J. Control. Release, 94, 423 (2004).

L142
GLYCOPEPTIDES PENETRATE THE BLOOD-BRAIN BARRIER: DRUGS FOR THE BRAIN FROM THE BRAIN.

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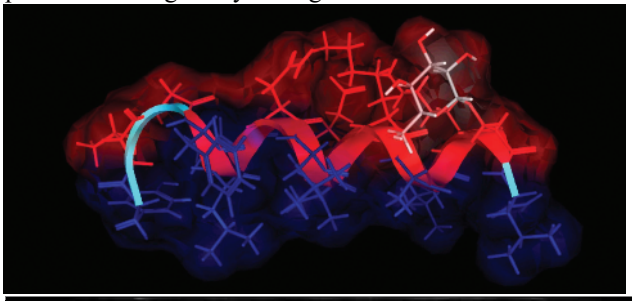
A series of glycopeptides based on the Leu-enkephalin analogue YtGFS*-$CONH_2$ (Lactomorphin®) led to greatly enhanced stability *in vivo* and effective penetration of the BBB. Transport through the BBB hinges on the *biousian* nature of the glycopeptides— the glycopeptides have two conflicting conformational manifolds, a H_2O soluble state, and an amphipathic state at H_2O-membrane phase boundaries. Multiple lines of evidence suggest that the BBB transport mechanism is absorptive endocytosis. Mixed μ/δ-agonists showed antinociceptive potencies greater than morphine, and lacked many of the side effects generally associated with classical μ-selective opiate analgesics. Lactomorphin® has been synthesized under cGMP conditions, and is undergoing pre-clinical drug safety testing.



The *biousian* design was extended to much larger glycopeptides related to β-endorphin (16 residues), which also penetrate the BBB and produced antinociception in mice. Plasmon waveguide resonance (PWR) studies showed that the amphipathic helices bind to membrane bilayers with μM to low nM K_D's. NMR studies indicate that the helicity increases as the solvent is changed from H_2O to H_2O/TFE to SDS micelles to bicelles. The presence of diverse endogenous neuropeptide transmitters and neuromodulators in the human brain is potentially applicable to the treatment of a wide range of behavioral disorders.

L143
NOVEL SUGAR-DERIVED MOLECULES INHIBIT ONCOGENIC RAS ACTIVATION

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Nearly 30% of all human tumors are linked to a mutation of the ras oncogene. The Ras proteins mediate cell proliferation by linking an extracellular growth factor with the nucleus via signal transduction involving an activation-deactivation cycle. Activation of non-mitogenic Ras-GDP to mitogenic Ras-GTP is reversible in healthy cell but is constantly switched on in mutated tumor cells. Inhibition of GDP-GTP nucleotide exchange of tumorigenic Ras could prevent continued cell growth and hence could represent e treatment for cancer. Design and synthesis of novel Ras inhibitors with a bicyclic core derived from the natural sugar D-arabinose are presented. Molecular modelling and NMR (STD and transfer-NOEs) studies showed that these molecules bind Ras near the Switch II region of the protein surface (Figure). As Switch II region undergoes conformational changes during the nucleotide exchange process, the binding of ligands in this point of the protein probably interferes with Ras activation-deactivation cycle. Data will be presented assessing that all synthetic compounds are active in inhibiting nucleotide exchange on p21 human Ras *in vitro* and two of them selectively inhibit Ras-dependent cell growth in mammalian tumor cells.

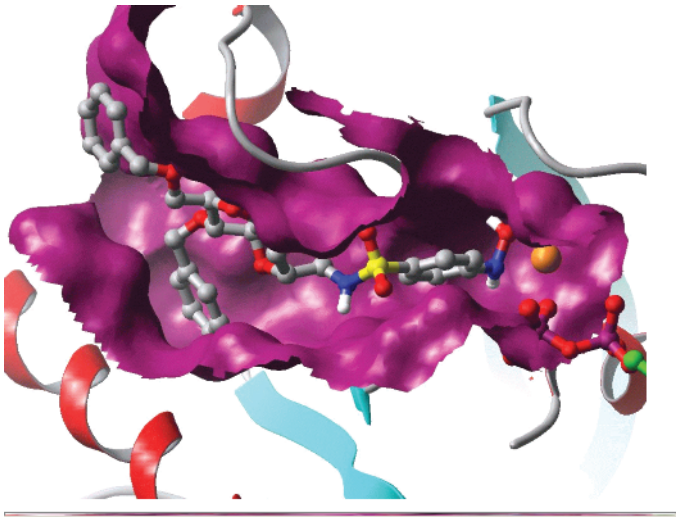

Figure: Sugar-derived synthetic inhibitor bound to Ras near to the Switch II region.

L144
TRANSFORMING GROWTH FACTOR BETA1 INDUCES XYLOSYLTRANSFERASE I EXPRESSION IN HUMAN CARDIAC FIBROBLASTS

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The extracellular matrix (ECM) plays an important role for the integrity and the function of the human heart. A severe and common disease of the heart is the dilated cardiomyopathy (DCM) which is associated with a fibrotic degeneration and a matrix deposition in the failing tissue. In the present study we analyzed the regulation of xylosyltransferase I (XT-I) the key enzyme in glycosaminoglycan biosynthesis in heart biopsies of DCM patients and mechanically stretched cardiac fibroblasts. We could show that patients with DCM exhibit significantly increased levels of XT-I in heart biopsies of the right and left ventricle. In failing hearts, raised XT-I levels were paralleled with an significantly increased XT-I enzyme activity and an elevated content of decorin. Primary cardiac fibroblasts subjected to cyclic mechanical stretch were used as a model system for the increased mechanical stress on the ventricular wall at DCM. For mechanically stretched fibroblasts we found significantly increased XT-I and collagen type I mRNA levels compared to controls. In cardiac fibroblasts we could also achieve an increased mRNA expression of both genes after treatment with recombinant TGF-β_1. Our results demonstrate that mechanical stress with elevated levels of TGF-β_1 induces XT-I expression in cardiac fibroblasts and might have impact for the myocardial remodeling in the dilated heart. Therefore, the quantification of XT-I is applicable for the assessment of tissue alterations.

L145
SULFATION OF HEPARAN SULFATE DEACREASES OVER TIME IN CEREBELLAR DEVELOPMENT

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Heparan sulfate proteoglycans (HSPGs) are implicated in morphogen signaling during central nervous system patterning, neurogenesis, axon guidance, migration, neurite outgrowth, and synaptogenesis. It is increasingly evident that HS structure changes in a highly dynamic manner. It has been described that HS is needed for the mitogenic activity of Shh, a growth factor for cerebellar neuronal precursors, at post natal day 6 (P6) but not at P3 or P9, indicating changes in HS synthesis (Rubin, *et al*., Development. 2002, 129(9):2223-32). We observed that CS/HS ratio in mice cerebella does not change significantly during the first two weeks of age, suggesting that the effects of HS on Shh activity would be due to modifications in HS structure, and not in its amount. Based on that, we looked for changes in the structure of cerebellar HS synthesized over time. Our data show that HS from P6 cerebellum has a greater sulfate content (greater amounts of N-sulfated and N-sulfated, 6-*O*-sulfated disaccharides), than HS from P3 and P9 cerebella, analyzed by heparitinase II degradation followed by ion exchange HPLC. Analysis of intact HS by mass spectrometry (ESI-MS) showed an increase in the amount of N-acetylated 6-*O*-sulfated disaccharide over time. On the other hand, the amount of a monosulfated tetrasaccharide (N-acetylated disaccharide, N-acetylated 6-*O*-sulfated disaccharide) does not change. Our data show that the sulfation profile of cerebellar HS changes over time, and these changes could be important to modulate the activities of Shh and other factors in the developing cerebellum. Supported by FADA-UNIFESP, FAPESP and CNPq.

L146
NEURITOGENIC AND GROWTH FACTOR BINDING ACTIVITIES OF THE BRAIN CHONDROITIN SULFATE/DERMATAN SULFATE HYBRID CHAINS

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Chondroitin sulfate (CS) and dermatan sulfate (DS) chains play roles in the central nervous system. Notably, CS/DS hybrid chains (E-CS/DS) purified from embryonic pig brains

bind growth factors and promote neurite-outgrowth toward embryonic mouse hippocampal neurons in culture. However, the neuritogenic mechanism is not well understood. Here, we show that pleiotrophin (PTN), a heparin-binding growth factor, produced mainly by glia cells, was the predominant binding partner for E-CS/DS in the membrane-associated protein fraction of neonatal rat brain. The CS/DS chains were separated on a PTN-column into unbound, low-affinity and high-affinity fractions. The latter two fractions promoted outgrowth of dendrite- and axon-like neurites, respectively, whereas the unbound fraction showed no such activity. The activity of the low-affinity fraction was abolished by an anti-PTN antibody or when glia cells were removed from the culture. In contrast, the high-affinity fraction displayed activity under both these conditions. Hence, PTN mainly from glia cells mediated the activity of the low-affinity, but not high-affinity, fraction. The anti-CS antibody 473HD neutralized the neuritogenic activities of both fractions. Interaction analysis indicated that the 473HD epitope and PTN-binding domains in the E-CS/DS chains largely overlap. The three affinity subfractions differed in disaccharide composition and the distribution of L-iduronic acid-containing disaccharides along the chains. Oversulfated disaccharides and non-consecutive iduronic acid-containing units were the requirements for the E-CS/DS chains to bind PTN and to exhibit the neuritogenic activities. Thus, CS subpopulations with distinct structures in the mammalian brain play different roles in neuritogenesis through distinct mechanisms at least in part by regulating the functions of growth factors.

L147
UDP-GLUCOSE DEHYDROGENASE AND ITS INVOLVEMENT IN GAG SYNTHESIS.

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INTRODUCTION: UDP-glucose dehydrogenase (UGDH) supplies the cell with UDP-glucuronic acid, a precursor of glycosaminoglycan (GAG) and proteoglycan synthesis. In this study, we report the cloning and the characterization of the UGDH from the amphibian *Xenopus laevis* (xUGDH) and the effects of its expression in human cells models. METHODS: We cloned xUGDH from commercial EST library and cloned it in expression vectors for bacteria and eucariotic cells. We transfected human cells with xUGDH or specific siRNAs for UGDH using nucleofector apparatus from AMAXA. The UDP sugars were assessed by CZE and hyaluronan (HA) and GAG were measured with electrophoretic method (FACE) and HPLC. Size of GAG was measure by gel filtration chromatography by HPLC.
RESULTS: We found that xUGDH is similar to other known UGDH sequences both at the genomic and the protein levels, including kinetic parameters. The up regulation of xUGDH mRNA in eucariotic cells is related with an increase of UDP-glucuronic acid in cytoplasm and with hyaluronan synthesis.
CONCLUSION: From our experiments we can suppose that UDP sugars, GAG precursors, influenced the hyaluronan synthesis in eucariotic cells, whereas the synthesis of other GAG was not affected, and this data can be justified considering that HA synthesis is performed by HA synthases that are enzymes on the cell membrane and take precursors from cytoplasms, whereas other GAG are synthesized by Golgi apparatus and therefore are indirectly influenced by UDP precursors.

L148
ENGINEERING OF AN ARTIFICIAL *O*-GLYCOSYLATION PATHWAY IN THE YEAST *SACCHAROMYCES CEREVISIAE* : PRODUCTION OF *O*-FUCOSYLATED EPIDERMAL GROWTH FACTOR-LIKE DOMAIN

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Humanizing attempts of yeast glycosylation pathway have been reported by several groups. However, there are no reports on the manipulation of protein fucosylation in yeast. Although fucose is not a general component of sugar chains in yeast, we have challenged for the construction of *in vivo O*-fucosylation system in budding yeast *Saccharomyces cerevisiae*. We already reported the efficient conversion of GDP-mannose to GDP-fucose in yeast using the co-expression system of *Arabidopsis thaliana MUR1* and *AtFX/GER1* genes. This sugar nucleotide pool was used for *in vivo O*-fucosylation through further expression of the other responsible genes, GDP-fucose transporter, protein *O*-fucosyltransferese and acceptor peptide. The EGF domain of human Factor VII was selected as an acceptor and engineered for its efficient secretion by the fusion with prepro alpha-factor via His6 tag sequence. The presence of fucose on secreted EGF domain was confirmed by fucose-specific lectin blotting and MALDI-ToF MS analysis. An efficient *O*-fucosylation of EGF domain also suggested the correct folding of 3 disulfides in EGF domain and its recognition by protein *O*-fucosyltransferase. This system enabled us to identify the uncharacterized membrane proteins assumed as GDP-fucose transporters and to examine the effect of fucose metabolic cycle on sugar nucleotide transporter activity. We confirmed that the glycoengineering of yeast to create *in vivo O*-fucosylation system is a powerful tool to identify the uncharacterized genes involved in *O*-fucosylation pathway and to analyse the structure and function relationship of *O*-fucosylated glycoproteins, for instance, in Notch receptor and ligand.

L149
DROSOPHILA FUNCTIONAL GLYCOMICS USING RNAI SYSTEM

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Currently, the elucidation of the functions of glycans is a topic of widespread interest and importance in cell and developmental biology. The glycosylation is performed in the Golgi apparatus by various glycosyltransferases, which transfer sugars from sugar-nucleotides to acceptor substrates. Sugar-nucleotide transporters supply a variety of sugar-nucleotides, which are synthesized in the cytosol, as donor substrates of glycosyltransferases in the Golgi apparatus.
Drosophila melanogaster is well established as a model for genetic analysis. Our molecular evolutionary study showed that a prototype of glycosyltransferases was conserved between mammals and *Drosophila*. RNA interference (RNAi) is becoming an efficient reverse genetic tool for studying gene function in model organisms. For analyses of the basic physiological functions of glycans, we applied the *Drosophila* inducible RNAi system to glycosyltransferases and sugar-nucleotide transporters.
Silencing in the whole fly body resulted in lethality to prove essential roles of glycans in development. Tissue-specific silencing induced various malformed phenotypes in the flies, by which genes were classified into some groups. These clusters might reflect the biosynthetic pathways and distinct functions of the various glycans. We will present a new approach to the comprehensive analysis of glycan function in development.

L150
PREDICTION OF THE 3D STRUCTURE OF A SCHISTOSOMA MANSONI OLIGOSACCHARIDE USING GENETIC ALGORITHMS AND MOLECULAR MECHANICS (MM3)

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The use of computational methods for conformational analysis of complex oligosaccharides is of great interest in many cases where experimental data are difficult to obtain. In the present work a newly developed genetic algorithm, GLYGAL [1], has been used to perform conformational search for oligosaccharides using energy evaluations with molecular mechanics MM3. The method has been applied on an oligosaccharide of the cercarial larvae of the parasite *Schistosoma mansoni*. The genetic algorithm was applied on the repeating unit which contains 5-6 sugar residues, essentially a LacdiNAc mainchain with a di- or trifucosyl side chain. Also two fucose residues at the non-reducing terminal were included in the calculations. The preferred conformation shows the di-/trifucosyl branch to curl up into the mainchain without making unfavourable interactions. The mainchain has an extended linear conformation. The two fucoses at the non-reducing end stick out into the bulk without any backfolding onto the mainchain. Further work will be needed to determine the flexibility of the fucosyl branches and the possible stabilizing effect they could have on the mainchain.
The conformational data that we have obtained are of interest for the rational design a vaccine against *Schistosoma mansoni*.
1) Nahmany, A.; Strino, F.; Rosen, J.; Kemp, G. J. L.; Nyholm, P.-G. 2005. The use of Genetic Algorithm Search for Molecular Mechanics (MM3) Based Conformational Analysis of Oligosaccharides., Carbohydrate Research, 340/5, 1059-1064.

L151
NEW DATABASE OF BACTERIAL CARBOHYDRATE STRUCTURES

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CSDB, a database containing data on natural carbohydrates with known primary structure, has been developed. In addition to the structure and bibliography, each record in the CSDB contains the abstract of the publication, data on the carbohydrate source, methods of structure elucidation, information on the availability of spectral data and assignment of NMR spectra when available, data on conformation, biological activity, chemical and enzymatic synthesis, biosynthesis, genetics and other related data. The key feature is the possibility to search within the database using as parameter a fragment(s) of the structure or indexed tags, including carbohydrate source, keywords and bibliography.
Currently, the CSDB contains about 7500 records on bacterial carbohydrates, including the corresponding part of CarbBank, a database developed at the end of XXth century (about 3000 records on structures reported before 1995). This coverage is approaching the total number of bacterial carbohydrate structures ever reported. Data from both literature and CarbBank have been checked for consistency prior to the upload and corrected when necessary.

The CSDB interface includes the web-based user part, web-based administrator part and programming gateways for automated data interchange. A possibility is provided for cross-referring to other databases containing information on natural carbohydrates. The CSDB is available at http://www.glyco.ac.ru for free usage and validated user data submission.
This work was performed within the framework of the ISTC Partner Project #1197, supported by the CTR Program of the US Department of Defense (ISTC Partner), and the RFBR grant 05-07-90099.

L152
GLYCODATABASES: WHAT IS ALREADY AVAILABLE? WHAT ARE THE NEXT STEPS?

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In comparison to the genomic and proteomic area, the compilation of data collections for carbohydrates and the development of appropriate bioinformatics tools have lagged behind. However, which the emergence of glycomics initiatives aiming to decipher new, so far unknown biological functions of glycans, the availability of comprehensive databases will be a prerequisite to successfully perform high-throughput *glycomics* projects. Consequently, the development of informatics tools as well as databases containing glyco-related data collections has gained momentum in recent years. The current status of the worldwide available resources will be reviewed.
Despite the increase of available data, the current situation in glycobiology is characterized by an extensive loss of primary experimental data. Most of these data are either not published (MS-spectra, HPLC-profiles and often NMR spectra) or are distributed in various journals using different notations, assignments and data formats.
The EUROCarbDB project aims to create the foundations for databases and
bioinformatics tools in the realm of glycobiology and glycomics, and will establish mainly the technical framework for bottom to top initiative where all interested research groups can feed in their primary data. The new infrastructure will constitute the nucleus for the creation of a depository for carbohydrate related data similar to the extensively used data collections in the area of genomics and proteomics.
The aims and current status of the project (www.eurocarbdb.org) will be presented.
EuroCarbDB is a Research Infrastructure Design Study Funded by the 6th Research
Framework Program of the European Union (Contract: RIDS Contract number 011952)

L153
SUGAR-BINDING PROPERTIES OF VIP36, AN INTRACELLULAR ANIMAL LECTIN OPERATING AS A CARGO RECEPTOR

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The vesicular integral protein of 36 kDa (VIP36) is an intracellular animal lectin, which acts as a putative cargo receptor recycling between the Golgi and the endoplasmic reticulum (ER). Although VIP36 interacts with glycoproteins carrying high mannose type oligosaccharides, detailed analyses of sugar-binding specificity discriminating isomeric oligosaccharide structures have not been performed yet. In the present study, we have analyzed sugar-binding properties of a recombinant carbohydrate recognition domain of VIP36 (VIP36-CRD) by frontal affinity chromatography (FAC) and NMR methods. For this purpose, a pyridylaminated sugar library, consisting 21 kinds of oligosaccharides including isomeric structures, was prepared and subjected to FAC analyses. NMR analysis revealed that VIP36-CRD preferentially binds the Manα1-2Manα1-2Man branch (D1 branch) of high mannose type oligosaccharides. The FAC data has shown that glucosylation and trimming of the D1 mannosyl branch interfere with the binding of VIP36-CRD. VIP36-CRD exhibits a bell-shaped pH dependence of sugar-binding with optimal pH around 6.5. By inspection of the specificity and optimal pH of sugar-binding of VIP36 and its subcellular localization, together with the organeller pH, we suggest that VIP36 binds glycoproteins retaining the intact D1 mannosyl branch in the *cis*-Golgi network and recycles to the ER, where it releases the cargos due to higher pH, thereby contributing to quality control of glycoproteins.

L154
GALECTIN PHYLOGENY, SPECIFICITY, CELLULAR EFFECTS, AND DEVELOPMENT OF NEW INHIBITORS

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We are comparing the properties of different types of galectins, a protein family with about 15 mammalian members, functionally implicated in inflammation and cancer. The one carbohydrate recognition domain (CRD) galectins-1, -2, -3 and -7, and each (CRD) of the bi-CRD galectins-4, -8 and -9, as well as selected mutants, have been analyzed for their binding specificity by a solution phase assay based on fluorescence polarization against a library of about 75 fluorescein tagged saccharide probes (tracers). Distinct and remarkable differences in the binding affinity among various galectin CRDs have been found, in particular, between the two CRDs within each bi-CRD galectin. Synthetic galectin inhibitors were designed and about 300 have been screened as inhibitors in the FP-assay against several of the 10 galectin CRDs being studied. Both compounds inhibiting many galectin CRDs and those more selective for one or two have been found. The best inhibitors so far have submicromolar K_d for galectin-3. The galectin specificity for natural and artificial ligands can be fairly well summarized by a model involving five subsites A-E, where site C is the most conserved and binds galactose whereas the others vary among the galectins. Cellular consequences of galectin specificity are indicated by the comparison of their cell surface binding (affinities and quantities), endocytosis, and fast responses such as agglutination and surface phosphatidyl serine exposure as measured by flow cytometry and other techniques. One emerging result is that the two CRDs of a bi-CRD galectin strongly synergize even if they have very different specificity.

L155
MULTIVALENT TN(GALNACα1→SER/THR), Tα(GALβ1→3GALNACα1→SER/THR) AND II(GALβ1→4GLCNAC) CONTAINING GLYCOTOPES AS IMPORTANT LIGANDS FOR A SERUM LECTIN, CBL FROM INDIAN CATFISH, *CLARIAS BATRACHUS*

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Several lectins bind tumor-associated carbohydrate antigens Tn and Tα. Polyvalency of Tn/Tα glycotopes plays a critical role in lectin-glycoconjugate interaction. To determine the clustering effect of Tn/Tα containing glycotopes on a vertebrate lectin, CBL from serum of *Clarias batrachus* was purified successively by ammonium sulphate precipitation, affinity chromatography on melibiose-Sepharose 4B column and anion-exchange chromatography on Res Q column. CBL is a monomeric lectin of subunit mass 70 kDa. CBL agglutinated human erythrocytes irrespective of any blood group and its activity was Ca^{2+} dependent. Carbohydrate-binding of CBL was investigated using a series of sugars and mammalian glycoconjugates by enzyme linked lectino-sorbent assay and lectin-glycan inhibition assay. CBL showed specificity for GalNAc/Gal as monosaccharide having preference for α-linkage. Hydrophobicity showed little influence over the binding as p-nitrophenyl-α-D-Gal was 1.3 times more potent inhibitor than Me-α-D-Gal. The shape of carbohydrate recognition domain is more fit with α-Gal containing di- and trimer than monomer since the inhibitory potency of stachyose, raffinose and melibiose were 9.27, 4.47 and 3.78 times more than α-D-Gal. Detailed study indicated that multi-valency or clustering effect of Tn (GalNAcα1→Ser/Thr), Tα (Galβ1→3GalNAcα1→Ser/Thr) or II (Galβ1→4GlcNAc) containing glycotopes were much more potent for interacting with CBL, which was 10^4 or 10^5 times more active than Gal or GalNAc. The affinity order of CBL for ligands was crypto-Tn or II > crypto-Tα > > > > > T (Galβ1→3GalNAc), II > Galα1→3/4/6Gal > α-GalNAc > α-Gal > β-GalNAc > β-Gal > Gal.

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L156
NMR STRUCTURAL BIOLOGY OF SUGAR-RECOGNIZING UBIQUITIN LIGASE INVOLVED IN GLYCOPROTEIN DEGRADATION

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Accumulating evidence shows that carbohydrate moieties determine the fates of glycoproteins in cells, i.e. folding, transport, and degradation, *via* interactions with a variety of intracellular lectins, e.g. ER chaperones, cargo receptors, and ubiquitin ligases. To gain insights into the mechanisms of the molecular recognition by those intracellular lectins, we performed NMR analyses of their carbohydrate-protein and protein-protein interactions.

Here we report a stable-isotope-assisted NMR study of molecular recognition of SCF^{Fbs1}, a sugar-recognizing ubiquitin igase complex that ubiquitinates misfolded glycoproteins for proteasomal degradation. Based on NMR spectral data, the amino acid and sugar residues involved in the carbohydrate-protein interactions were identified [1]. Inspection of NOE data allowed us to to characterize ligand-lectin contacts at atomic resolution. We revealed that the carbohydrate recognition domain (CRD) of SCF^{Fbs1} binds the carbohydrate-polypeptide junction of its substrates, which is hidden in a native glycoprotein but is likely to be exposed in unfolded glycoproteins doomed to proteasomal degradation. The exposed sugar-polypeptide junction could be a target of cytoplasmic peptide:N-glycanase (PNGase), which contribute to the proteasomal degradation of glycoproteins by removal of their *N*-glycans. It was shown that the CRD inhibits deglycosylation of denatured ribonuclease B by PNGase *in vitro*, suggesting that SCF^{Fbs1} protects substrates during polyubiquitination from attack by PNGase. The solution structure and interaction of the PUB domain of mouse PNGase will also be presented.

1. T.Mizushima et al. Nature Struct. Mol. Biol. ***11,*** *365-370 (2004)*

L157
INSIGHT INTO STRUCTURE - FUNCTION RELATIONSHIPS OF *RALSTONIA SOLANACEARUM* LECTINS

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A number of pathogen microorganisms utilize lectin-carbohydrate interaction to recognize and infect host organisms. The comprehension of the molecular mechanisms which gives a pathogenic bacterium the ability to invade, colonize and reorient the physiopathology of its host is a goal of primary importance to direct the conception of new strategies to fight against these pathogenic agents. *Ralstonia solanacearum* is a soil-born bacterium, which is responsible for bacterial wilts on more than 200 plant species including potato, tomato, banana and other economically important corps. *R. solanacearum* infects its hosts beginning with the root system and presents a very strong tropism for the xylem vessels. Its extensive multiplication in the water-conducting system leads to a systemic infection of the plant.
Three lectins RSL (9.9 kDa) [1], RS-IIL (11.6 kDa) [2] and RS20L (20 kDa) have been found in *R. solanacearum* extract and purified using affinity chromatography. All lectins were crystallized by vapor diffusion and high and ultra-high (0.94Å resolution of RSL/α-methylfucoside) resolution data were collected at ESRF, Grenoble, France. RSL is a trimer that generates a six-bladed β-propeller with high affinity for fucose. RS-IIL is a tetramer with calcium-mediated and binds mannose. The same specificity is reported for RS-20L which is the first reported bacterial galectin . The structural data have been supplemented by titration microcalorimetry and surface plasmon resonance studies defining lectins specificity to various oligosaccharides including those that could be the target for the lectins in plants.

[1] Kostlánová, N. et al., J Biol Chem, in press
[2] Sudakevitz, D. et al., Mol. Microbiol., 52, 691 (2004)

L158
GALECTIN-3 PROMOTES EPITHELIAL CANCER CELL ADHESION TO ENDOTHELIUM BY INTERACTION WITH CANCER-ASSOCIATED MUC1, VIA TF ANTIGEN

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Galectin-3, a key member of the naturally-occurring galactose-binding galectins family, shows increased serum concentration in cancer patients that correlates with cancer spread. MUC1 is a large trans-membrane mucin protein that is over-expressed and shows reduced expression of the complex *O*-glycans and increased expression of the oncofetal Thomsen-Friedenreich (Galβ1,3GalNAc-α, TF) disaccharide and sialyl-TF tri-saccharide in epithelial cancer cells. We report here that cancer-associated MUC1 is a novel and natural ligand of galectins-3. Recombinant galectin-3 at a concentration (0.5-1 μg/ml) similar to the five-fold elevation that is found in the serum of patients with breast or gastrointestinal cancer caused significant (97% and 80%, respectively) increase of adhesion of MUC1 expressing-ZR-75-1 human breast epithelial cancer cells and -HT29-5F7 human colon cancer cells to human umbilical vein endothelial cells (HUVEC). Galectins-3 selectively enhanced cell adhesion of MUC1 transfected HCA1.7+ human breast epithelial cells, whose MUC1 carries predominantly unsubstituted TF antigen, but not of MUC1 transfected MDE9.2+ ras-transformed canine kidney epithelial-like cells, whose MUC1 carries predominantly sialyl-TF structure. Pre-treatment of the MDE9.2+/- cells with *A ureafaciens* sialidase, which completely removed the terminal sialic acid residues on sialyl-TF of MUC1 in MDE9.2+ cells, resulted in significant increase (103%) of MDE9.2+, but not of the revertants MDE9.2-, cell adhesion to HUVEC by galectin-3. These results suggest that galectins-3-MUC1 interaction, largely via TF, promotes epithelial cancer cell adhesion to endothelium.

As epithelial cancer-associated MUC1 typically carries much more copies of TF antigen than MUC1 on benign epithelial cells, binding of circulating galectin-3 to cancer-associated MUC1 in the bloodstream may represent a critical step in the metastatic cascade of epithelial cancers.

L159
CEREBROSIDES ARE INCREASED IN THE DIABETIC RETINA: IMPLICATIONS FOR DIABETIC RETINOPATHY

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Diabetic retinopathy is a debilitating complication of diabetes and a leading cause of vision loss; however the fundamental mechanisms contributing to vision loss remain undefined. Dysregulated sphingolipid metabolism has been associated with insulin resistance and cellular death in many model systems and diseases. It is thus hypothesized that diabetes alters sphingolipid metabolism contributing to neuronal pathologies in diabetic retinopathy.
Ceramide content as well as ceramide metabolites were measured after 2, 4 and 8 weeks of streptozotocin (stz)-diabetes by ESI/MS/MS. After 4 and 8 weeks of diabetes there was a ~30% decrease in total ceramide content, concomitant with a significant ~30% increase in monohexosylceramides (cerebrosides) levels in fed diabetic rats compared to their age-matched controls. Acute insulin therapy as well as a short-term lowering of glucose via fasting did not affect the augmented flux through the glycosphingolipid biosynthetic pathway. By immunohistochemistry, we demonstrate that glucosylceramide synthase localizes in many layers of the retina with strongest immunoreactivity in the photoreceptor outer segments. Lastly, treating R28 retinal neurons with N-butyldeoxyglactonojirimycin, a glucosylceramide synthase inhibitor, leads to increased insulin sensitivity when measuring the activation state of p70 S6K, a prosurval kinase whose activity is decreased in diabetic retinas.
It is speculated that an increase in cerebrosides, and possibly higher order glycosphingolipids could contribute to the pathogenesis of diabetic retinopathy by contributing to insulin resistance resulting in neuronal cell death.

L160
AN ABERRANT N-GLUCOSYLATION TRIGGERING AUTOIMMUNITY IN MULTIPLE SCLEROSIS

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Our research is focused on the characterization of a disease form of Multiple Sclerosis (MS) mediated by autoantibodies. We demonstrate, for the first time that an aberrant N-glucosylation is a fundamental determinant of autoantibody recognition in Multiple Sclerosis. Thus, we developed CSF114(Glc), an antigenic probe accurately measuring IgM autoantibodies in sera of a patients' population, as disease biomarkers. CSF114(Glc), a structure-based designed glycopeptide, is able to recognize, by ELISA, the presence of specific IgM autoantibodies in sera of a Multiple Sclerosis patients' population, but not in blood donors, and other autoimmune conditions. Autoantibodies specific for CSF114(Glc), isolated from Multiple Sclerosis patients, recognized myelin and oligodendrocyte antigens by immunohistochemistry, but not other non relevant tissues. We demonstrate that CSF114(Glc) is a reliable, specific probe in a longitudinal study of untreated Multiple Sclerosis patients. Development of IgG/IgM anti-CSF114(Glc) antibodies paralleled clinical activity, and brain lesions positive to MRI. Therefore, a CSF114(Glc)-based immunoassay on sera may have important prognostic value in monitoring Multiple Sclerosis disease progression guiding the optimal therapeutic treatment. These specific biomarkers of the disease are instrumental to characterize (by a proteomic approach) the corresponding native post-translationally modified proteins involved in triggering the autoimmune response.
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L161
GLYCOCONJUGATED COLLAGEN CROSS-LINKS: THE FIRST SYNTHESIS OF GLUCOSYL-GALACTOSYL-PYRIDINOLINE, A BIOLOGICAL MARKER OF SYNOVIUM STATUS

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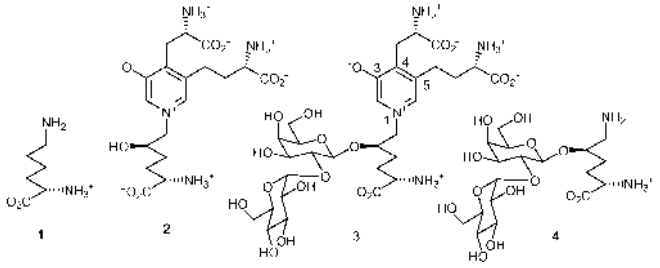
Collagen, during the maturation, suffers some posttranscriptional modifications ending with the formation of intra and intermolecular cross-links. During bone remodelling and collagen degradation, some cross-linked collagen fragments are excreted unchanged in human urine. Some of them are used as reliable biochemical markers of total

collagen turnover, useful for the diagnosis of osteoporosis and other bone diseases. Some of them, as hydroxylysine 1 and pyridinoline 2 (Py), appear to have a different meaning in their glycoconjugated form (glucosyl-galactosyl-pyridinoline; glc-gal-pyr) 3 and glucosyl-galactosyl-hydroxylysine (glc-gal-hyl) 4 respectively.



In particular, the levels of glc-gal-pyr 3 appear to be strongly predictive markers of the progression of joint destruction in various joint diseases, especially in some types of particularly aggressive rheumatoid arthritis.
We have performed the first total synthesis of the optically pure glc-gal-pyd 3, in amounts suitable for biological experiments. Our own protocol involves the preparation of a β-O-glycosylated hydroxylysine synthon and its assembling with an appropriate bromo ketone, in a "one pot" reaction affording the masked glc-gal-pyd, deblocking of which affords the desired glc-gal-pyd 3. The preliminary preparation of the starting glycosylated protected hydroxylysine was reached using an original protocol which allowed to obtain also the glycosylated hydroxylisine 4.

L162
ST6GAL-I CONSTRUCTS AND MASKS GLYCAN LIGANDS FOR B CELL AND MACROPHAGE LECTINS THAT CONTRIBUTE TO HUMORAL IMMUNITY AND AUTOIMMUNE DISEASE

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The immune system generates and responds to endogenous post-translational protein modifications that are topologically separated, with phosphorylation confined to intracellular compartments and glycosylation occurring predominantly in the secretory pathway. We find that the ST6Gal-I sialyltransferase generates cell surface glycan counter-receptors for the B cell transmembrane CD22 Siglec glycoprotein that reduce co-localization with the antigen receptor and lower the threshold for protein phosphotyrosine induction in immune signaling. More generally, this finding reveals that cell surface glycan linkages controlled by glycosyltransferase enzymes can modulate the assembly and disassembly of cell surface receptor complexes as a means of regulating intracellular signal transduction. ST6Gal-I further selectively masks ligands for cell asialoglycoprotein receptors on Kupffer cells that prevent rapid clearance of serum immunoglobulin-M and diminished functional levels. Depressed humoral immunity due to ST6Gal-I deficiency reflects enforced glycan changes typically restricted to post-activated B cells that are capable of attenuating the development of Lyn-deficient autoimmune disease. The glycoprotein binding specificities of endogenous Siglec and C-type lectins comprise mechanisms integrating protein glycosylation, phosphorylation, and homeostasis in pathways of immune regulation.

L163
ANTI-NOR ANTIBODIES REPRESENT A NEW TYPE OF ANTI-αGal ANTIBODIES PRESENT IN HUMAN AND ANIMAL SERA

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Human sera contain various anti-αGal antibodies besides the anti-Galα1-3Gal which are best known. Recently, we characterized new common antibodies which agglutinate rare erythrocytes with inherited NOR characteristics. The NOR erythrocytes contain unique glycosphingolipids (globoside extention products) terminating with a Galα1-4GalNAcβ1-3Gal- unit. The specificity of anti-NOR antibodies was tested with a panel of synthetic oligosaccharides (ELISA), including Galα1-4GalNAcβ1-3Gal (NOR-tri) and Galα1-4GalNAc (NOR-di), synthesized by Dr. T. Norberg and coworkers (Uppsala, Sweden), and an analog of NOR-tri, Galα1-4Galβ1-3Galβ1-4Glc (Gal_3Glc), synthesized by Dr. N.V. Bovin and coworkers (Moscow, Russia). Agglutination of NOR erythrocytes was strongly inhibited by NOR-tri; therefore, anti-NOR antibodies were affinity-purified from several serum samples on a NOR-tri-HSA-Sepharose 4B column. The purified antibodies were found to show two types of fine specificity. Type 1 antibodies reacted strongly with NOR-tri and Gal_3Glc, and weakly with NOR-di and Galα1-4Gal. Type 2 antibodies reacted strongly with NOR-tri and NOR-di, and weakly with Gal_3Glc and Galα1-4Gal. Anti-NOR antibodies did not react with Galα1-3Gal. The two kinds of epitopes recognized by human anti-NOR antibodies can be presented as Galα1-4GalNAcβ1-3Gal (type 1 shadowed, type 2 in frame) In some human serum samples either type 1 or type 2 antibodies dominated, while other samples contained both types in various proportions. In contrast to anti-Galα1-3Gal, anti-NOR antibodies were found in rabbit and horse sera. The animal antibodies were also purified on the NOR-tri-HSA-Sepharose 4B column. They detected the NOR glycolipids on the TLC plates, as did the human anti-NOR. Moreover, the

animal anti-NOR antibodies tested so far also showed the type 1 or type 2 specificity, i.e. they recognized either the Galα1-4GalNAc(or Gal)β1-3Gal- or Galα1-4GalNAc- unit, respectively. The presence of natural anti-NOR antibodies in human and animal sera suggests that Galα1-4GalNAc-containing antigens are more frequent in nature than currently thought.

L164
GALα(1,3)GAL SYNTHESISED BY iGb3 SYNTHASE ELICITS ANTIBODIES IN MICE WITH A DELETION IN THE α1,3GALACTOSYLTRANSFERASE GENE

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Introduction. A critical step towards the clinical reality of xenotransplantation is the removal of the carbohydrate xenoepitope Galα(1,3)Gal to prevent hyperacute rejection of transplanted pig organs. Unexpectedly, pigs and mice with a deleted α1,3galactosyltransferase (αGT) gene still express Galα(1,3)Gal showing that α1,3GT is not the only enzyme synthesizing this epitope. We showed that iGb3 synthase (iGb3S) synthesises Galα(1,3)Gal on lipid in α1,3GT -/- mice. Our aims were to examine whether α1,3GT -/- mice can mount an antibody response to iGb3 lipid and to determine the molecular mechanism of presentation.
Approaches and Results. Human 293 embryonic kidney cells were transfected with α1,3GT or iGb3S cDNAs and were used to immunise either α1,3GT -/- or (α1,3GT -/- x CD1 -/-) mice. Anti-Galα (1,3)Gal antibody levels were measured by ELISA. Strong anti-Gal antibody responses were observed in α1,3GT -/- mice immunized with α1,3GT-293 cells. An anti-Gal response was also observed in mice immunized with iGb3S-293 cells. Non-immunized mice contained very low levels of anti-Gal antibodies and this was not increased by immunization with 293 cells. Similar responses to immunisation with the three cell lines were observed in (α1,3GT -/- x CD1 -/-) mice. All antibody responses were ablated by CD4 treatment of the recipients.
Conclusions. Galα (1,3)Gal synthesized by iGb3S is immunogenic in α1,3GT -/- mice. The antibodies are T cell dependent and CD1 independent, suggesting an alternate uncharacterised pathway for glycolipid presentation. Similarly elicited antibodies may affect the survival of pig transplants into humans.

L165
CHARACTERISATION OF THE MOLECULAR BASIS FOR SEROTYPE SPECIFIC EXPRESSION OF LOS IN *MORAXELLA CATARRHALIS*

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Introduction: *Moraxella catarrhalis* is a human pathogen that causes serious diseases such as otitis media in children and lower respiratory ailments such as chronic obstructive pulmonary disease (COPD) in the elderly. Although the lipooligosaccharide (LOS) from *M. catarrhalis* is a virulence factor for this organism, there is a paucity of information regarding the mechanism of its assembly or the enzymes involved in its biosynthesis. Recent reports have identified three genes responsible for LOS biosynthesis in *M. catarrhalis* in Serotype B (1, 2).
Experimental approach: Mutant strains expressing truncated LOS were constructed using insertional inactivation. In brief: the genes encoding the putative glycosyltransferase were disrupted by insertion of a selectable marker (kanamycin resistance) into the gene of interest. The inactivated gene was inserted into *M. catarrhalis* and the bacteria cultured. LOS from the mutant bacteria were then extracted using a phenol-chloroform-pet ether extraction method with modifications (3,4) and were structurally characterised by mass spectrometry and NMR.
Results and Conclusions: This information has enabled us to assign a function to each gene encoding a glycosyltransferase. We have identified a similar locus to that recently reported (1, 2) and have additionally identified a fourth gene in the locus in strains of all serotypes, and a fifth gene in serotype A and C strains. In total fives genes implicated in LOS biosynthesis in *M. catarrhalis* were identified (*lgt*1, *lgt*2, *lgt*3, *lgt*4 and *lgt*5) encoding for glycosyltranserases. Interestingly, the presence/absence of *lgt*5 and the allele of *lgt*2, determined the serotype-specific LOS biosynthesis.

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L166
STRUCTURAL AND IMMUNOCHEMICAL STUDIES ON SPHINGOLIPIDS OF THE OPPORTUNISTIC MYCOPATHOGEN *Aspergillus fumigatus*

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Aspergilli are one of the most common fungal species which humans are exposed, and it is now recognized as the most prevalent airborne fungal pathogen in developed countries. Among this specie the *Aspergillus fumigatus* is considered the most pathogenic. Thus there exists a compelling interest in the discovery novel functional molecules and the pathways of new targets for development of antifungal therapeutics.
Acidic glycosphingolipid components were extracted from the *Aspergillus fumigatus* strains 237 and 9197and identified as glycosylinositol phosphorylceramides (GIPCs). By a combination of 1- and 2-D ^{1}H-nuclear magnetic resonance (NMR) spectroscopy, electrospray ionization mass spectrometry (ESI-MS), and gas chromatography/mass spectrometry (GC/MS), the structures of five major components were elucidated as:
Af-2: Man*p*(α1-3)Man*p*(α1-2)InsPCer
Af-3a: Gal*f*(β1-6)Man*p*(α1-3)Man*p*(α 1-2)InsPCer
Af-3b: Man*p*(α1-3)[Gal*f*(β1-6)]Man*p*(α1-2)InsPCer
A component containing a branching Gal*f*(β1-6) residue, essentially identical to Af-3b, was previously isolated from the dimorphic mycopathogen *Paracoccidioides brasiliensis*, but component Af-3a has a novel isomeric glycosylinositol structure not previously identified in any fungal GIPCs. Sera of patients exhibiting aspergillosis and mAb MEST-1 reacted strongly with Af-3a, -3b and -4 confirming the high antigenicity of fungi glycolipids containing terminal residues of galactofuranose. Af-4 is an additional novel component, possible bearing Gal*f*(β1-6) units on Man*p*(α1-3) residues of the common Af-2 core structure. Fine structural studies on Af-4 is underway.
Supported by: CAPES, CNPq, FAPESP, Neose, NIH

L167
ROLE OF *Leishmania (Leishmania) amazonensis* AMASTIGOTE GLYCOSPHINGOLIPIDS IN MACROPHAGE INFECTIVITY

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Previously we have demonstrated that mAb ST-3 directed to *Leishmania (Leishmania) amazonensis* amastigote glycosphingolipids (GSLs) inhibited about 80% of macrophage infectivity by amastigotes (Straus et al., 1993). Amastigote GSLs were purified by a combination of acetylation/deacetylation, Florisil and DEAE Sephadex chromatography, the neutral GSLs fraction, free of parasite LPGs and GIPLs, was utilized for micelle preparation by sonication. The β-D-Gal-globotriaosylceramide (Gal*p* β1-3Gal*p* *α*1-3Gal*p* β1-4Glc*p* β1-1Cer), the smallest GSL antigen recognized by ST-3, was purified by HPLC.
In this work it was demonstrated that the disaccharide Gal*p* β1-3Gal*p* inhibits the ST-3 binding to purified β-D-Gal-globotriaosylceramide and the macrophage infectivity by amastigotes, in a dose dependent way (at 1mM it inhibits about 48%).
The macrophage receptor for β-Gal-globotriaosylceramide and other structurally related GSLs from *L. (L.) amazonensis* amastigotes was analyzed by Western blotting of resident peritoneal mouse macrophage proteins fractionated by SDS-PAGE and transferred to nitrocellulose membrane. The nitrocellulose membranes were then incubated with micelles containing purified *L. (L.) amazonensis* GSLs or intact amastigotes. Fixed amastigotes or micelles containing highly purified parasite GSLs bound to a doublet protein of 30 kDa and were visualized in the nitrocellulose membrane using mAb ST-3.
These results clearly indicate that amastigote stage-specific GSL antigens play a key role in *L. (L.) amazonensis* binding to macrophages. The macrophage 30 kDa doublet, a putative amastigote GSL receptor, is 0.5% Triton X-100 insoluble membrane component, present in the macrophage surface. Detailed characterization of this macrophage receptor/lectin is underway.
Supported by CAPES, CNPq, FAPESP and PRONEX.

L168
RECENT PROGRESS IN UNDERSTANDING PROTEIN GLYCOSYLATION AND ITS COUPLING TO TRANSLOCATION

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Following initiation of translocation across the membrane of the endoplasmic reticulum via the translocon, polypeptide chains are N-glycosylated by the oligosaccharyl transferase (OT) enzyme complex. Translocation and N-glycosylation are concurrent events and would be expected to require juxtaposition of the translocon and the OT complex. To determine if any of the subunits of the OT complex and translocon mediate interactions between the two complexes, we initiated a systematic study in budding yeast using the split-ubiquitin approach.

Interestingly, the OT subunit Stt3p was found to interact only with Sec61p, while another OT subunit, Ost4p, was found to interact with all three components of the translocon, Sec61p, Sbh1p and Sss1p. The OT subunit, Wbp1p was found to interact very strongly with Sec61p and Sbh1p and weakly with Sss1p. Other OT subunits, Ost1p, Ost2p, and Swp1p were found to interact with Sec61p and either Sbh1p or Sss1p. We were able to confirm these split-ubiquitin findings by a chemical cross-linking technique.
Based on our findings using these two techniques, we conclude that the association of these two complexes is stabilized via multiple protein-protein contacts. Based on extrapolation of the structural parameters of the prokaryotic Sec complex to the eukaryotic complex, we propose a simple working model to understand the contacting proteins of the translocon-OT supercomplex.

L169
REGULATION OF LIPID-LINKED OLIGOSACCHARIDE BIOSYNTHESIS BY ER STRESS RESPONSES.

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Introduction: Lipid-linked oligosaccharides (LLOs) are the precursors of asparagine-linked glycans on glycoproteins. Defective LLO synthesis can therefore interfere with glycoprotein folding, causing endoplasmic reticulum (ER) stress and triggering the Unfolded Protein Response (UPR). Our recent fluorophore-assisted carbohydrate electrophoresis studies show that LLO biosynthetic intermediates are surprisingly abundant in mouse tissues. This implies that the LLO pathway does not operate at full capacity, and that regulation is required to maintain a balance between intermediate and mature LLOs. Since LLO defects can trigger the UPR, we have tested the hypothesis that the UPR is a "functional" regulator of LLO biosynthesis.
Approaches: Using mammalian cells, specific ER stressors (tunicamycin, dithiothreitol, thapsigargin) were used to examine potential roles both of known UPR effects (transcriptional regulation and translation attenuation) and novel UPR effects on LLO biosynthesis, by approaches including gene disruption and RNA interference for UPR stress sensors.
Results: 1) At least one LLO transferase mRNA is regulated in a classical manner, responding within hours of application of ER stress. 2) Translation attenuation modulates the turnover of LLOs, responding within 30-60 min. 3) By an unknown ER stress system, within 20 min. hexose-P is mobilized (most likely from glycogen) as a supplemental source of LLO precursors.
Conclusions: LLO biosynthesis appears to be controlled by "functional" UPR regulation. This involves both of the known UPR effects, and one novel mechanism. Further, these mechanisms are governed by the duration and strength of ER stress.
Supported by NIH grant GM38545 and Welch grant I-1168.

L170
BIOLOGICAL FUNCTIONS OF CORE FUCOSYLATION

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The core fucosylation (alpha1,6-fucosylation) of glycoproteins is widely distributed in mammalian tissues, and is altered under pathological conditions. Recently, deletion of the core fucose from IgG molecule has been reported to enhance antibody-dependent cell cytotoxicity (ADCC) activity up to 50 to 100-fold and therefore this antibody is very useful for the antibody therapy against cancer. To investigate physiological functions of the core fucose, we generated alpha1,6-fucosyltransferase (*Fut8*)-null mice, and found that disruption of *Fut*8 induces severe growth retardation and death during postnatal development. Histopathological analysis revealed that *Fut8*$^{-/-}$ mice showed emphysema-like changes in the lung, which were verified by a physiological compliance analysis. Biochemical studies indicated that lungs from *Fut8*$^{-/-}$ mice exhibit a marked overexpression of matrix metalloproteinases (MMPs), such as MMP-12 and MMP-13, previously associated with lung destructive phenotypes, and a down-regulation of extracellular matrix (ECM) proteins such as elastin. These changes are consistent with a deficiency in transforming growth factor-beta1 (TGF-beta1) signaling. In fact, *Fut8*$^{-/-}$ mice have a marked dysregulation of TGF-beta1 receptor activation and signaling, as assessed by TGF-beta1 binding assays and Smad2 phosphorylation analysis. Furthermore, exogenous TGF-beta1 potentially rescued emphysema-like phenotype, and concomitantly reduced MMP-12 expression in Fut8$^{-/-}$ lung. According to these results, we propose that core fucosylation of TGF-beta1 receptors is crucial for the formation and maintenance of the appropriate lung ECM architecture and suggest that perturbation of this function could underlie some cases of human lung emphysema.

L171
N-LINKED GLYCOPROTEIN DISLOCATION AND DEGRADATION: THE ROLE OF PEPTIDE:N-GLYCANASE

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Within the ER lumen, newly synthesized N-linked glycoproteins are co-translationally appended with a $(GlcNAc)_2Man_9Glc_3$ tetradecasaccharide. They associate with

lectins such as calnexin and calreticulin, chaperones that retain the glycoproteins in the ER until properly folded. Misfolded polypeptides are shuttled to ER membrane protein complexes that dislocate them into the cytosol where they are ubiquitinated, deglycosylated and ultimately degraded by the proteasome. Deglycosylation is mediated by peptide:N-glycanase (PNGase), a cysteine-based hydrolytic enzyme that cleaves high mannose glycans from glycosylated asparagine side chains in unfolded polypeptides. A high throughput screen identified the caspase inhibitor, z-VAD-fmk, as a potent inhibitor of PNGase. Using MALDI-MS, we have mapped the site of PNGase modification by z-VAD-fmk and show that adduct formation occurs exclusively at the putative active site cysteine. Use of this cell-permeable inhibitor has facilitated the separation of dislocation, deglycosylation and proteolytic steps. For substrates such as α_1-antitrypsin and TCRα, dislocation is coupled to deglycosylation and proteasomal degradation on the cytosolic face of the ER membrane. For MHC class I heavy chains, the processes of dislocation, deglycosylation and proteolysis occur in the cytosol and are uncoupled. We also find that inhibition of PNGase with Z-VAD-fmk does not prohibit proteasomal proteolysis. Thus, multiple pathways of degradation are available to the cell for removal of unwanted N-linked glycoproteins.

L172
INOSITOL DEACYLATION BY BST1P IS REQUIRED FOR THE QUALITY CONTROL OF GLYCOSYLPHOSPHATIDYLINOSITOL-ANCHORED PROTEINS IN YEAST

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Misfolded proteins are recognized in the endoplasmic reticulum (ER), transported back to the cytosol and degraded by the proteasome. A number of proteins are processed and modified by the glycosylphosphatidylinositol (GPI) anchor in the ER, but the quality control mechanisms of GPI-anchored proteins remain unclear. Here, we report on the quality control mechanism of misfolded GPI-anchored proteins. We have constructed a mutant form of β-1,3-glucanosyltransferase Gas1p (Gas1*p) as a model of the misfolded GPI-anchored protein. The Gas1*p that was modified with a GPI anchor but retained in the ER was degraded rapidly via the proteasome. After attachment of GPI to proteins, the acyl group on inositol of GPI moiety is removed in the ER. Disruption of *BST1* encoding GPI inositol-deacylase caused a delay in the degradation of Gas1*p. This delay was due to the effect of the deacylation activity itself of Bst1p. Disruption of genes involved in GPI-anchored protein concentration and *N*-glycan processing exhibited different effects on the degradation of Gas1*p and a soluble misfolded protein, CPY*. Further, Gas1*p associated with both Bst1p and BiP/Kar2p, a molecular chaperone, *in vivo*. Our data suggest that GPI inositol deacylation plays important roles in the quality control and ER-associated degradation of GPI-anchored proteins.

L173
LACTOSYLCERAMIDE :AN EMERGING LIPID SECOND MESSENGERAND ITS POTENTIAL ROLE IN ANGIOGENESIS

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Lactosylceramide is a member of the sphingolipid family and plays a critical role as a precusror to several key glycosphingolipids.In addition , the signaling role of LacCer has been shown recently in mediating growth factor and oxidized LDL induced cell proliferation in atherosclerosis and polycystic kidney disease, tumor necrosis factor induced cell adhesion, cholesterol efflux, locomotor function and inflammation. Thus, LacCer by its own right ,is emerging as a novel lipid second messenger in health and disease.Herein, we present evidence suggesting that LacCer may well play a critical role in Vascular endothelial growth factor(VEGF) -induced angiogenesis.
First, using siRNA for LacCer synthase(GalT-V) we show that the ablation of LacCer synthase gene in human endothelial cells markedly inhibited VEGF -induced angiogenesis. Second, using pharmacological inhibitors of GlcCer synthase and LacCer synthase such as PPMP and D-PDMP we show that VEGF induced angiognenesis was completley mitigated in endothelaial cells but was reversed by exogenous supply of LacCer but not other GSL.Finally, using mutant endothelial cells that lack platelet endothelial cell adhesion molecule(PECAM-1) we show that LacCer induced angiogenesis critically requires the expression of PECAM-1. In sum, our findings elaborate a novel pathway wherein VEGF targets GalT-V to generate LacCer. In turn, LacCer recruits PKC,PLA-2 and NfKB as down -stream signaling intermediates that collectively induce PECAM-1 expression and angiogenesis.

L174
GENERATION AND CHARACTERIZATION OF MOUSE MONOCLONAL ANTIBODIES TO DETERGENT INSOLUBLE MEMBRANES ISOLATED FROM HL60 AND PC12 CELLS

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Glycosphingolipids and cholesterol form lipid microdomains in the plasma membranes. The microdomains were isolated biochemically as detergent-insoluble membrane (DIM) by sucrose density-gradient centrifugation of nonionic detergent lysate. We previously reported the presence of phosphatidylglucoside (PtdGlc)-based lipid microdomain distinct from the sphingolipids-based domains in the plasma membranes of HL60 cells. In order to understand the biological roles of these microdomains, we have isolated 50 clones of mouse monoclonal antibodies (MAbs) against the DIM fractions from HL60 and PC12 cells. We found that some of MAbs recognized proteins such as annexin 1, aminopeptidase N, and acrogranin. Other MAbs reacted with lipid antigens. Thin layer chromatography (TLC) / immunostaining assay revealed the presence of three types of MAbs preferentially reacting to PtdGlc, lysophospholipids, respectively, and specifically recognizing GM1a/GD1b. Immunocytochemical analysis revealed that these MAbs except for MAb against lysophosphatides well stained cell surface membranes, probably lipid microdomains. Furthermore some MAbs had potential to induce cell proliferation or differentiation. These results showed that MAbs against DIM fraction is one of valuable tools for the study of functional roles of lipid microdomains.

L175
GLYCOSPHINGOLIPID METABOLISM AND PACLITAXEL RESISTANCE IN HUMAN OVARIAN CANCER

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Anticancer drugs typically induce programmed cell death or apoptosis. In some cases, apoptosis is mediated by generation of cellular ceramide. Cancer cells develop multiple mechanisms that allow them to become resistant to chemiotherapeutic angents. The dysfunctional metabolism of ceramide is a mechanism that contribute to cell drug resistance.
Paclitaxel is an antimitotic agent used in the treatment of a number of major solid tumors, particularly in breast and ovarian cancer. Paclitaxel treatment is able to induce apoptosis in a human ovarian carcinoma cells by an increase in the cellular ceramide levels and a decrease in the sphingomyelin levels, due to the activation of sphingomyelinases.
Since apoptosis often represents the final common event of chemotherapy-induced cell death, insight into the mechanisms of drug resistance can be gained from a better understanding of the pathway of drug-induced apoptosis in tumor cells. Indeed, defects in the apoptotic pathway have been observed to confer insensitivity to the cytotoxic effects of chemotherapy and may therefore represent an important mechanism for the development of chemoresistance.
The acquisition of the paclitaxel-resistant phenotype is accompanied by unique alterations in the complex sphingolipid pattern. Specifically the resistant cells show a modified ganglioside pattern respect to sensitive cells. The main gangliosides from resistant cells were identified as GM3. Changes in the expression of gangliosides could contribute to the onset of a resistant phenotype in tumor cells.

L176
CELL SURFACE BETA 1, 4-GALACTOSYLTRANSFERASE 1 PROMOTES APOPTOSIS BY INHIBITING THE EGFR SIGNALING PATHWAY

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Overexpression of β1,4-galactosyltransferase1 (β1,4GT1) enhances the SMMC-7721 hepatocarcinoma cell susceptibility to cycloheximide (CHX)-induced apoptosis. We provided that cell surface β1,4GT1 was responsible for the increased apoptosis. When cells were transfected with the truncated long form β1,4GT1 (TL), which displaces the endogenous cell surface β1,4GT1 from its cytoskeletal attachment, the apoptosis was not increased. Previous studies have shown that intracellular galactosyltranslation is unaffected in cells expressing either the full-length or TL proteins. This suggests that the pro-apoptotic effect of cell surface β1,4GT1 may have no relation with its catalytic activity. We also found that long form β1,4GT1 interacted with EGFR in vitro and in vivo. Long form β1,4GT1 inhibited EGFR autophosphorylation at Tyr1068 particularly during CHX induction. It also inhibited PKB/Akt phosphorylation at Thr308 and Ser473, Erk and JNKs phosphorylation. Some of the Bcl-2 family proteins that are regulated by PKB/Akt, Erk and JNKs were also affected by long form β1,4GT1 during apoptosis. Phosphorylation of Bcl-2 at Ser70, which was catalyzed by JNKs, was decreased in long form β1,4GT1 transfected cells. Translocation of pro-apoptotic protein Bad and Bax to the mitochondria which was regulated by PKB/Akt and Erk, was also increased in long β1,4GT1-overexpressing cells. The changes of Bcl-2, Bad and Bax were consistent with that of PKB/Akt, Erk and JNKs. Thus we conclude that cell surface β1,4GT1 promotes apoptosis by inhibiting EGFR-mediated apoptotic signaling pathway. Therefore, the mechanism of pro-apoptotic role of β1,4GT1 is defined.

L177

COLD TEMPERATURE-INDUCED MODIFICATIONS TO THE LIPOPOLYSACCHARIDE STRUCTURE AND BIOLOGICAL PROPERTIES OF *Yersinia pestis*

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The natural environmental temperatures for *Y. pestis* vary from 0 to 40ºC. Significant variations in structure and bioactivity of the LPS samples from bacteria cultivated at 25-28ºC or 37ºC suggest a role for variation in overcoming the defense systems of both warm-blooded mammals (host) and cold-blooded insects (vector). Here we studied the LPS from strain KM218 grown at 6ºC (LPS-6) to mimic the conditions in animals during winter hibernation.
The composition and strucutre of LPS-6 and derived core oligosaccharide and lipid A samples were determined by GLC fatty acid analysis, high-resolution electrospray ionization MS, and NMR spectroscopy. Two types of LPS-6 molecules were found, which differ in fatty acid composition, the presence or absence of 4-amino-4-deoxyarabinose in lipid A and phosphoethanolamine in the core as well as in the occurrence of either galactose or D-*glycero*-D-*manno*-heptose in the core.
A decrease of growth temperature from 25ºC to 6°C caused a significant reduction in resistance of KM218 to polymyxin B. The TNF-α-inducing activity *in vitro* and toxicity in mice of LPS-6 were similar to those of LPS-37, but LPS-6 induced a lower TNF-α production and was less toxic than LPS-25.
Therefore, cold temperature changed significantly the LPS structure and biological properties of *Y. pestis*. These modifications may reflect an armistice between the bacterium and the host during winter hibernation, allowing the bacteria to persist asymptomatically.
This work was performed within the framework of the ISTC Partner Project #1197, supported by the CTR Program of the US Department of Defense (ISTC Partner).

L178
DISSOCIATION OF INSULIN RECEPTOR AND CAVEOLIN COMPLEX BY GM3: A NEW PATHOLOGICAL FEATURE OF INSULIN RESISTANCE IN ADIPOCYTES

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Membrane microdomains (lipid rafts) are now recognized as critical for proper compartmentalization of insulin signaling. We demonstrated previously that in the state of insulin resistance of adipocytes induced by TNF alpha, the inhibition of insulin metabolic signaling and elimination of insulin receptor (IR) from the caveolae microdomains was caused the accumulation of ganglioside GM3 (1, 2). We were able to extend these *in vitro* observations to living animals using obese Zucker *fa/fa* rats and *ob/ob* mice, in which the GM3 synthase mRNA and GM3 levels in the white adipose tissues are significantly higher than in their lean controls. To insight into the molecular interaction of IR, caveolin-1 and GM3, we have examined the affinity of these molecules by immunoprecipitation and live cell imaging utilizing FRAP (fluorescence recovery after photobleaching) and TIRF (total internal reflection fluorescence). We found that IR forms complex with caveolin-1 and GM3 independently; and in the GM3 enriched membranes, the mobility of IR was increased by dissociating IR-caveolin interaction. Thus, we propose a new pathological feature of insulin resistance in adipocytes caused by dissociation of IR-caveolin-1 complex through the interaction of IR with GM3 in microdomains.
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L179
DIFFERENTIAL EXPRESSION OF HSP-70, HSP-90 AND GRP-74/Bip, GRP-94 IS A KEY TO TUNICAMYCIN-INDUCED APOPTOSIS IN CAPILLARY ENDOTHELIAL CELLS.

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Tumor remodeling is a dynamic process and requires angiogenesis. Reports from our laboratory have indicated a link between increased $Glc_3Man_9GlcNAc_2$-PP-Dol (LLO) and angiogenesis due to increased DPMS activity, increased expression (~1.5-fold) of HSP-70 & HSP-70, and reduced GRP-70/Bip expression with no change in caspase-3,-8, or –9 activity or the Bcl-2 expression. The objective of the present

study is to establish the molecular mechanism of the cross talk between LLO biosynthesis and angiogenesis. We report here that tunicamycin, an inhibitor of GPT activity in the ER not only inhibited the capillary endothelial cell proliferation but arrested cells in G1 phase within 32 hours and induced apoptosis. Reduced expression of Bcl-2, cyclins D1 & D2 and their catalytic partner CDK4, and increased DNA laddering, annexin-V binding as well as increased expression of caspase-3, 9- and –12 all supported it. Protein synthesis inhibitor, cycloheximide had no effect. However, tunicamycin caused a significant reduction (~87%) in LLO biosynthesis and cell surface N-glycan expression, a 134-426% increased expression of GRP-78/Bip & GRP-94 with no effect on HSP-70 & HSP-90. To explain the "ER stress" the intracellur protein structure was analyzed by FT-IR spectroscopy. The results indicated a gradual shifting of Amide I band (typical of C = O stretching at 1600-1700 cm^{-1}) to a higher frequency in tunicamycin treated cells. We, therefore, conclude that alteration of protein structure could occur as early as 3h of tunicamycin treatment to induce unfolded protein response (UPR)-mediated apoptotic cell death of capillary endothelial cells. Partly supported by DAMD17-03-1-0754; U54-CA096297.

L180
CD22-LIGAND INTERACTIONS AFFECT MICRODOMAIN LOCALIZATION AND SIGNALING

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CD22 is a negative regulator of B cell signaling, an activity modulated by its interaction with glycan ligands containing α2-6 linked sialic acids. B cells from mice deficient in the enzyme forming the CD22 ligand (ST6-/-) exhibit suppressed BCR signaling. Here we report that mice deficient in both CD22 and its ligand (CD22-/- ST6-/-) exhibit restored BCR signaling, demonstrating that the suppressed signaling of cells from ST6-/- mice is mediated through CD22. Coincident with suppressed BCR signaling, we find that B cells of ST6-/- mice exhibit a net redistribution of sIgM to clathrin rich microdomains containing the majority of CD22, resulting in a 2-fold increase in co-localization of CD22 with the BCR. The altered microdomain localization appears to be mediated by CD22, since B cells of CD22-/- ST6-/- mice exhibit wild type sIgM distribution. To further enable the assessment of cell-surface *cis* ligand interactions, we developed a novel method for *in situ* photoaffinity crosslinking of glycan ligands to CD22. Suprisingly, sIgM and other glycoproteins that bind to CD22 *in vitro* do not appear to be major *cis* ligands of CD22 *in situ*. Instead, CD22 appears to recognize glycans of neighboring CD22 molecules as *cis* ligands, forming homo-multimeric complexes. Taken together these data indicate that association of CD22 with the BCR does not require and may even be reduced by *cis* ligand interactions.

L181
REGULATING EFFECT OF LIF ON THE EXPRESSION OF FUT7 -PROBE INTO THE MECHANISM OF SLE^X ATTENDING IMPLANTATION

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Implantation is a complex developmental process regulated through the interaction of many implantation-related factors. Plenty of studies have paid attention to the fucosylated oligosaccharides (such as H_1, Le^X, Le^Y, sLe^X) which highly expressed by embryo and uterus during pre-implantation. Recently it has reported that trophoblast L-Selectin and its ligand sLe^X on uterine epithelial cells mediate the adhesion at the maternal-fetal interface during implantation. In present study, the effect of LIF (a crucial factor of implantation) on the expression of FUT7 (fucosyltransferase VII, a key enzyme of sLe^X fucosylation) was analyzed by PCR, indirect immunofluorescence and immune-dot blotting to investigate the correlation between sLe^X oligosaccharide and LIF and probe into the mechanism of the role of sLe^X in implantation. Our results showed that LIF up-regulated FUT7 gene and enzyme expression on both of the maternal and fetal sides in dose-dependent manner (10ng/ml was the optimum); anti-LIF antibody had a reverse effect; the effect of LIF on the secretion of sLe^X in embryo medium showed the same tendency. Our study also found that the adhesion ratios decreased in the embryos and uterine epithelial cells co-culture model when sLe^X antigen on blastocysts surface blocked by antibody. In summary, sLe^X expression is affected by LIF via FUT7, and thus it involves in the regulation network of implantation; sLe^X which was expressed on the surface of blastocysts and uterine epithelial cells and both side may involve in mediating the embryo adhesion during implantation.
supported by National Natural Science Foundation of China (3027329 to Q Yan and 3017266 to ZM Zhu)

L182
SIALIC ACID LINKAGE REGULATION DETERMINES CARCINOMA DIFFERENTIATION IN VIVO

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Aberrant glycosylation and increased sialylation are hallmarks of cancer. One example is the enhanced expression of N-glycan alpha2-6-sialylation, which is associated with tumor progression and poor prognosis. The responsible enzyme is beta-galactoside:alpha2-6-sialyltransferase (ST6Gal-I). However, evidence for a role in tumor behavior is circumstantial, or based on cell culture experiments. We examined the role of ST6Gal-I in growth and differentiation of spontaneous mammary cancers in mice carrying an MMTV-promoter-driven polyoma middle-T antigen transgene. Tumors in wild-type animals were compared with those in mice either null for ST6Gal-I, or over-expressing it in epithelial cells (a Keratin-18 promoter-driven ST6Gal-I transgene). No change in tumor latency or growth rate occurred in either genetically modified background. Notably, mammary tumor cells in wild-type mice already expressed high levels of ST6Gal-I, as demonstrated by *Sambuccus nigra* agglutinin staining. In contrast, ST6Gal-I null tumors were negative for this staining, and appeared more differentiated, making glandular structures containing secretions. Immunohistochemistry showed expression of mammary epithelial differentiation markers and milk production. Muller and colleagues showed that beta1-integrin expression is critical for initiation of mammary tumorigenesis, and for maintaining the proliferative state of tumor cells (Cancer Cell. 6:159-70, 2004). Meanwhile, Bellis and colleagues showed that increased alpha2-6 sialylation of beta1-integrins on cultured carcinoma cells enhanced binding to type 1 collagen, and stimulated cell motility (Cancer Res. 11: 4645-52, 2005). Thus, we hypothesize that, while alpha2-6 sialylation of beta1-integrins enhances conformational changes promoting activation during tumor progression, the lack of this linkage in ST6Gal-I null mice reduces beta1-integrin function and favors differentiation.

L183
SEARCHING FOR TUMOR-ASSOCIATED GLYCOSPHINGOLIPIDS IN MALIGNANT HUMAN PANCREATIC TISSUE

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The prognosis of patients with pancreatic cancer is extremely poor, with 5-year survival rates lower than 5%. As yet no chemotherapeutic agent has produced a substantial survival benefit. Changes in the composition and structure of cell surface glycosphingolipids (GSLs) during neoplastic transformation have been reported. The aim of this study was to examine the level of expression of three CD-classified GSLs in human pancreatic cancer as potential therapeutic targets.

Cancerous tissue samples (n=12) and corresponding control specimens were obtained from the same patients that have undergone surgery. Tissues were homogenized and extracted with chloroform/methanol mixtures. After saponification of phospholipids, the extracts were separated by thin-layer chromatography (TLC). Comparative TLC immunostaining assays were performed with monoclonal anti-CD60a (GD3) and polyclonal anti-CD75s (IV^6nLc4) and anti-CD77 (Gb3) antibodies.

The highest percentage of enhanced GSL expression was detected for CD77 (Gb3). More than half (58%) of the pancreatic tumors investigated showed an enhancement of CD77(Gb3), 17% identical and 25% reduced expression compared to the corresponding tumor-free tissues. Broad positive reactions were observed for CD75s (IV^6nLc4) in 42% of the tumors compared to negative control tissues. Remaining 58% of the cancerous as well as the healthy tissues were negative. Clear positive expression of CD60a (GD3) was detected in 36% of the tumors, showing negative values for the corresponding controls and the remaining 64%.

Increased expression of certain GSLs detected in a random collection of pancreatic tumors renders these cell surface molecules excellent candidates for oncological applications, i.e., as targets for humanized monoclonal antibodies (CD60a), Shiga toxin (CD77) or viscumin (CD75s).

L184
SUBCELLULAR LOCALIZATION OF I-MBP AND ITS FUNCTIONAL IMPLICATION

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Mannan-binding proteins (MBPs) are C-type lectins specific for mannose, N-acetylglucosamine and fucose. Human MBP is encoded by a single mRNA but occurs naturally in two forms with differential post translational modifications: serum MBP (S-MBP) and intracellular MBP (I-MBP). It is well known that S-MBP has important roles in innate immunity and activates complement via the lectin pathway. On the other hand, the function of I-MBP is less understood, although we reported that I-MBP was present in the lumen of the ER and Golgi apparatus some years ago (1). Recently, functions of ER lectins with similar specificity have been the subject of intensive study with regards to the quality control and the transport of glycoproteins from ER to Golgi.

In the present study, as an extension of our previous study, human MBP cDNA was transfected into human hepatoma cells and the subcellular localization of I-MBP was examined using green fluorescent protein (GFP) fusions by confocal microscopy and electron microscopy. I-MBP showed distinct accumulations in cytoplasmic granules, and predominantly localized in ER and COPII vesicle-mediated ER-Golgi transport route. Upon treatment of the wild-type MBP transfectants with tunicamycin or the trasfection of a mutant MBP with no sugar binding activity, the unique intracellular distribution of I-MBP disappeared and displayed a diffuse cytoplasmic localization. These findings strongly suggest that the I-MBP may play important roles in glycoprotein intracellular transport.

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L185

SIGNALING OF CEACAM1, A MAJOR CARRIER OF LEWISX STRUCTURES IN GRANULOCYTES, IS REGULATED BY CELL DENSITY AND PARTITIONING INTO LIPID RAFT-LIKE STRUCTURES.

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Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1/CD66a) is expressed in leukocytes, epithelial and endothelial cells. Recent reports have suggested that CEACAM1 behaves as a signal transduction molecule that affects cell behavior by regulating proliferation, migration, apoptosis and morphogenesis. Furthermore it acts as a receptor for pathogens, like *Neisseria meningitis* or *Haemophilus influenca*, while mouse CEACAM1 was found to bind the murine hepatitis virus. In our studies we analyzed the structural-functional relationship of the extra- and intracellular CEACAM1 part. We could show that different members of the CEA family are the major carrier of LewisX structures in human granulocytes. By mass spectrometric methods, we clearly identified different LewisX epitopes on CEACAM1-bound N-glycans, which are currently analyzed for ligand specificitiy. In another attempt we analyzed the CEACAM1-mediated signaling and found that CEACAM1 is associated with different intracellular proteins, such as Filamin A, src-family tyrosine kinases (SFK) and protein tyrosine phosphatases. The activation state and formation of CEACAM1 signaling complexes depends on cell density. Because SFKs are asscociated with lipid raft membrane microdomains and CEACAM1 tyrosine phosphorylation was cholesterol-depletion sensitive, we speculated that CEACAM1 might also be targeted to membrane microdomains. By cholesterol depletion of cellular membranes with methyl-β-cyclodextrin (MβCD) and by sucrose density centrifugation experiments after cell lysis with the detergent CHAPS, we clearly showed the localization of this receptor within lipid raft-like membrane microdomains. In conclusion, we demonstrated novel mechanism how signaling of this highly glycosylated, LeX-containing adhesion receptor is regulated.

L186

ENZYMATIC REMODELING OF FATTY ACYL CHAINS OF GLYCOSPHINGOLIPIDS TO ANALYZE THE FUNCTIONS OF PUFA-CONTAINING GM1A ON LIPID MICRODOMAINS AND SIGNALING

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Polyunsaturated fatty acids (PUFAs) are widely distributed in the glycosphingolipids (GSLs) as a minor constituent, however their physiological functions have not been clarified. Here, we report the remodeling of fatty acyl chains of GSLs by SCDase[1)] and the analysis of functions of PUFA-GSLs on the lipid microdomains and signaling. We found that α-linolenic acid-containing GM1a (C18:3-GM1a), but not stearic acid (C18:0) or oleic acid (C18:1)-containing GM1a, induced apoptosis in mouse neuroblastoma Neuro2a cells, *i.e.* intranucleosomal DNA fragmentation, chromatin condensation, and caspase activation were observed when the cells were cultured with C18:3-GM1a at a concentration of 40 μM. C18:3-GM1a was found to be concentrated in lipid microdomains of Neuro2a cells after the removal of loosely bound GM1a by bovine serum albumin. The treatment of Neuro2a cells with C18:3-GM1a seemed to affect the structure of microdomains estimated by the behavior of NCAM-120, possibly leading to apoptosis through the decreased phosphorylation level of ERK1/2 but not that of SAPK/JNK. These findings and the ubiquitous distribution may indicate the physiological significance of PUFA-containing GSLs *in vivo* and suggest why naturally occurring GSLs do not possess PUFAs as a major constituent.

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L187

INCREASED SUBSTRATUM ADHESION AND β1 INTEGRIN EXPRESSION IN HUMAN COLON CANCER CELLS TRANSDUCED WITH α2,6 SIALYLTRANSFERASE (ST6Gal.I)

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β-galactoside α2,6 sialyltransferase (ST6Gal.I), the enzyme which adds sialic acid in α2,6-linkage on lactosaminic termini of glycoproteins, is frequently overexpressed in cancer. This change is associated with increased malignancy but the molecular bases of this relationship remain elusive. In this study, we have investigated the phenotypic changes related with overexpression of α2,6-sialylated lactosaminic chains by using clones derived from transfection of the human colon cancer cell line SW948 with the ST6Gal.I cDNA or mock transfected. Compared with untransfected cells and mock-transfectants, the ST6Gal.I-expressing clones show increased adherence to fibronectin and collagen IV but not to hyaluronic acid. Neuraminidase treatment resulted in reduced binding to fibronectin and collagen IV of a ST6Gal.I-expressing clone but in no effect on a mock-transfectant. While untransfected and mock-transfected cells tend to form multistratified tissues, ST6Gal.I-expressing clones show a flatter morphology and the tendency to grow as a monolayer. FACS analysis revealed that all ST6Gal.I-expressing clones display higher amounts of β1 integrins on the cell surface; this difference is not supported by differences at the β1 integrin mRNA level and is lost when cells are left in suspension for several hours, suggesting a mechanism of membrane stabilization of β1 integrins dependent on the presence of α2,6-linked sialic acid. Our data support a model in which the presence of α2,6-linked sialic acid on membrane glycoconjugates increases the binding to extracellular matrix components, resulting in membrane stabilization of β1 integrins, further strengthening the binding. This mechanism provides a basis for the altered phenotype associated with ST6Gal.I overexpression.

L188
REGULATORY MECHANISM OF SELECTIN LIGAND EXPRESSION LEVEL: CONTROL OF LEUKEMIC CELL EXTRAVASATION BY LENTIVIRAL SHRNA FOR TRANSCRIPTIONAL ACTIVATOR SP4

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B cell precursor acute lymphocytic leukemia (BCP-ALL) cells are characterized by their ability of extravascular infiltration, in which process surface selectin ligands are involved. In the present study, we have demonstrated in BCP-ALL cells that the expression of *core 2 β1,6-N-acetylglucosaminyltransferase-1 (C2GnT1)* transcript correlated well with that of selectin ligand, and that selectin ligand expression and the leukemic cell extravasation were regulated by Sp4 through *C2GnT1* transcription.
The promoter region containing an Sp1 binding consensus was shown to be important in *C2GnT1* transcription, and disruption of the Sp1 site resulted in a 65% reduction of transcriptional activity. Reporter assay revealed that *C2GnT1* transcription was activated mainly by Sp4. Endogenous *Sp4* was markedly expressed in BCP-ALL and pre-B cells, but not in mature B cells. Introduction of *Sp4* significantly induced *C2GnT1* activation in a Sp4(-)/C2GnT1(-)/B cell line and *Sp4* gene knock-down by lentiviral shRNA system resulted in sLeX down-regulation in a Sp4(+)/sLeX(+)/B cell line. Extravasation ability of the leukemic cells was assayed by transplantation of cells into γ-irradiated NOD/SCID mice followed by flowcytometric analyses. The immigrant cell number was significantly reduced in spleen, liver and bone marrow when *Sp4* gene expression was knocked-down by lentiviral shRNA.
These results indicate that glycosyltransferase responsible for selectin ligand-capped glycan synthesis (C2GnT1) and its transcriptional regulator (Sp4) are involved in the extravasation process through regulation of selectin ligand expression. These results suggest that Sp4 may be a candidate of effective therapeutic targets for prevention of extravascular invasion of BCP-ALL cells.

POSTERS

Analytical procedures for glycoconjugates

P001
CHROMATOGRAPHIC CHARACTERISATION OF BACTERIAL OLIGOSACCHARIDES BY ANION EXCHANGE PROFILES COMBINING DIFFERENT DETECTION TECHNIQUES

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Bacterial polysaccharides often plays an important role in the virulence of the organism and their covalent coupling to protein carriers is being successfully employed to develop effective vaccine against different relevant diseases. Consequently, in the production process of last generation glycoconjugates vaccines, a detailed characterisation of oligosaccharides involved in the conjugation reaction is required.
Anion exchange chromatography traditionally is an important tool to characterise oligosaccharides and chromatographic separations are usually obtained using hydroxide-acetate based eluents onto polymeric strong anion exchangers. In this work different eluent ions and chromatographic phases were investigated, combining both electrochemical and UV detection after hydroxide elimination by micro membrane suppressor. Two different approaches were considered: the evaluation of low capacity resins and the use of a stronger anion eluent, obtaining information about molecular weight distribution of an high polymerisation level pool of polyphosphate and polycarboxylate oligosaccharides.

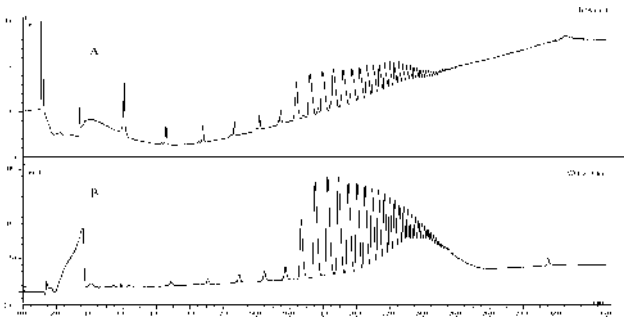

Figure 1: HPAEC profiles of polyphosphate oligomers
A: Integrated amperometric detection
B: UV-Vis detection (200nm)

P002
GLYCOSYLATION OF GOAT FOLATE BINDING PROTEIN PROBED BY MASS SPECTROMETRY

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Introduction
The folate-binding proteins (FBPs) and the folate receptors are members of a structural family of proteins sharing a high level of sequence similarity. These proteins are a major focus of research as targets for tumor therapy, among other reasons, because they participate in the transport of antifolate drugs as well as in that of their natural ligand. All of the members of this group have in common the presence of a variable number of glycosylation sites required for efficient protein folding.
Although the goat FBP sequence differs from the better known bovine FBP in only 8 positions, one of the three glycosylation sequons present in bovine FBP is absent and another is predicted to have a very low substitution efficiency. We report here the amino acid sequence of goat FBP and the structure of the carbohydrates attached to its only glycosylation site.
Experimental approaches
The intact oligosaccharides were released from the glycoprotein, separated from the peptides and directly analysed by MALDIMS leading to oligosaccharide profiling. PSD experiments led to detailed structural information on the sequence of antennae and branching pattern of the glycoforms.
Results and conclusions
The structural characterisation of the glycans from goat FBP was achieved by using an integrated approach based on MS methodologies. Several glycoforms bearing fucosylated and non complex type oligosaccharides with a different number of sialic acids were identified. Moreover, hybrid and high mannose structures were also detected. The only glycosylation site in this protein, believed to be essential for efficient protein folding, was thus completely characterized .

P003
MASS SPECTROMETRY OF GLYCOPROTEINS

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Introduction
Glycosylation is an extremely important co- and post-translational modification able to modulate protein function as

it can determine activity, stability, localisation and turnover of the protein itself. Glycans modulate protein function and influence folding, biological lifetime and recognition of binding partners. Unlike the core proteins, glycans are expressed as a set of variations on a core structure, are polydisperse in nature and are usually branched structures with the monomers linked together at different positions. The structural characterisation of glycoproteins is complicated by the intrinsic heterogeneity of these molecules due to the presence of several different structures at the same glycosylation site.

Experimental approach

The intact sugar moieties are released from the glycoprotein separated from peptides, and directly analysed by MALDIMS, leading to oligosaccharides profiling. Detailed structural information on the sequence of the antennae and the branching pattern was obtained by PSD experiments. Finally digestion of the glycopeptide by exoglycosidase array and subsequent MS assay of the digestion products led to sequencing and a linkage-form determination .

Results and conclusions

The entire structural characterisation of the oligosaccharide moiety from different human and recombinant proteins, namely recombinant EPO, kiwi PME and human GP17, including the branching pattern, the sequence of the antennae, the position of the residues in the chains, and the definition of the anomericity was accomplished by using an integrated approach based on biochemical procedures and MS technologies.

P004

SULFATIDE MODIFICATIONS AND QUANTITATIONS BY MASS SPECTROMETRY IN ALZHEIMER DISEASE.

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Sphingoglycolipids (SGLs) are important constituents of the nervous system. We have shown previously by ESI tandem mass spectrometry (Colsch et al.,2004), after purification of the several SGLs species, that adult mouse brain contains ceramides from galactocerebrosides and sulfatides (sulfogalactosylceramides) with both sphingosine and sphingadienine.

The presence of these 2 sphingoid bases add to the complexity of these compounds already known by the many fatty acids present in their molecular isoforms. Sulfatides have been reported to be decreased in temporal lobe of Alzheimer brain with very mild dementia (Han et al, 2002) ; we used a similar methodology to the one we used previously, to compare sulfatides, in different areas of brain, at a late stage of the disease.

Electrospray ionization appears to be a rapid method for the analysis of the major brain lipids (Han and Gross, 2005). It does not require any lipid purification before mass spectrometry. Nevertheless, the minor isoforms of sulfatides can not be detected, such as those containing sphingadienine for which a previous purification is necessary. Quantitation has been possible now using a specific internal standard, C17:0/d18:0 sulfogalactosylceramide.There was a clear reduction of sulfatide isoforms mainly in the prefrontal area of brain from patients with a severe level of Alzheimer's disease. The reduction was less important in the temporal lobe. The respective decrease was 30% and 15%. We are in the course of developing a correlation between sulfatide level and the degree of brain damage and to determine whether these alterations are specific for Alzheimer's disease.

P005

SEPARATION AND STRUCTURAL ANALYSIS OF GLYCOPEPTIDES WITH ISOMERIC N-GLYCANS FROM HUMAN SERUM IGG BY USING NORMAL-PHASE LIQUID CHROMATOGRAPHY/ESI-IT-TOF MS AND MSN SPECTRAL LIBRARY

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Glycosylation is one of the most common forms of posttranslational modifications in eukaryotic proteins. During the last decades, mass spectrometry (MS) based analysis of glycopeptides has become a topic of high interest to elucidate glycosylation sites and site heterogeneity. However, it is still a difficult and complex task, in particular, due to the structural microheterogeneity of carbohydrate moieties.

In this presentation, we propose a new approach for glycopeptide analysis using normal-phase liquid high performance chromatography (NP-HPLC)/ESI-IT-TOF MS and CID MSn spectral library of 2-aminopyridine derivatized oligosaccharides (PA N-glycans). A reverse-phase (e.g., ODS or porous graphitized carbon (PGC) column) chromatography is normally used for separation of glycopeptides. However, glycopeptides attaching several types of isomeric N-glycans seem to be difficult to sufficiently separate. This also makes the following MS based structural analysis complicate. Recently, several methods used normal-phase (e.g., amide or hydroxyethyl column) chromatography for separation of oligosaccharides and glycopeptides have been reported. We also applied a kind of normal-phase liquid chromatography for separation of isomeric PA N-glycans (e.g., fucosylated N-glycans and fucosylated bisecting N-glycans) from human serum IgG, which are difficult to separate on the reverse-phase (ODS) chromatography, and succeeded to completely separate them using a gradient elution of ammonium acetate buffer (pH= 6.8) and acetnitrile. From CID MS3 spectrum of B-type

fragment ion cleaved between chitobiose glycoside bond (GlcNAcβ1-4GlcNAc) of oligosaccharide in each MS2 spectrum and MSn spectral matching, these N-glycan isomers were easily identified. A usefulness of this method is shown by investigating other glycopeptides with isomeric N-glycans.

P006
STRUCTURAL ANALYSIS OF GLYCOPEPTIDES USING NANOHPLC/ESI-IT-TOF MS AND MS^N SPECTRAL MATCHING

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During the past decade, a mass spectrometry (MS) has been used intensively for the rapid and sensitive analysis of glycoproteins (i.e., glycoproteomics), in combination with various glycosidase and protease treatments (digestions). However, such the MS(or MS^n) based glycoproteomics still remains many difficulties and challenges due to a microheterogeneity of the attached oligosaccharides including their binding site, a complexity of their structural analysis and a lack of proper database of oligosaccharides.
In this presentation, we propose a new approach to analyze both the peptide sequence and the oligosaccharide (N-glycan) structure of glycopeptides, directly and simultaneously. This approach is based on CID MS^n (n=1-3) spectra acquired by ESI-IT TOF MS in the positive-ion mode, MS^3 spectra of peptide fragment ions, and MS^n spectral matching for MS^3 spectra of oligosaccharide fragment ions. We first optimized CID conditions for MS^2 so that a doubly-protonated precursor ion of glycopeptide on MS^1 spectrum causes efficiently a glycoside-bond cleavage between chitobiose residue (GlcNAcβ1-4GlcNAc) attached to asparagine (Asn) and also, two protons (H^+) of the precursor ion are shared by both the resulting fragments (i.e., B-type fragment ion of oligosaccharide and peptide ion remaining GlcNAc residue on Asn). Then, MS^3 spectra derived from these fragment ions, the peptide sequence including N-glycan binding site and the structure of the fragmented B-type ion of N-glycan can be obtained. A preliminary test of this approach using several glycopeptides of RNase B and Ovalbumin, provided promising results, especially, for the structure of N-glycan based on MS^n spectral matching.

P007
NOVEL ISOTOPE CODED DERIVATIZATION REAGENTS FOR HIGHLY SELECTIVE AND QUANTITATIVE GLYCAN PROFILING

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Introduction Though quantitative mass spectrometry using stable isotope coded reagents has emerged as a powerful tool for differential protein analysis, their application to glycomics analysis has been scarce. Development of novel stable isotope coded reagents suitable for glycan will accelerate the protein post-translational modification analysis. In this study, we reported the synthesis of novel glycan specific derivatization reagents with stable isotope tag and relative quantitation of derivatized *N*-glycan profiling on MALDI-TOF analysis.
Experimental Two novel derivatization reagents (aoWR(H) and aoWR(D)) were synthesized using tryptophanyl-arginine as a scaffold. N-terminal of dipeptide was used for the incorporation of oxylamino functionality, while C-terminal was methyl esterficated to generate a stable isotope tag (d_3-methyl). For standard derivatization, 10 μL of 2 mM reagent in 50 mM acetate buffer and 1 μL of oligosaccharide were mixed and then incubated at 90 °C for 1 h. After cooling the reaction tube, the reaction mixture was accordingly mixed with DHB and 0.5 μL of the resulting mixture was subjected to MALDI-TOF Mass analysis.
Results The newly stable isotope labeled derivatization reagents allowed a rapid and sensitive *N*-glycan profiling. Owing to its high ionization efficiency of aoWR on MALDI-TOF analysis, aoWR derivatives could be highly selectively detected with minimum purification steps. By incorporating mass differences of 3 Da between stable isotpe coded reagents, relative quantitation of derivatized *N*-glycan profiling between the two tissues were determined at one try. Furthermore, the MALDI-TOF/TOF analysis of aoWR(H) and aoWR(D) derivatized oligosaccharides gave very intense fragment signal at *m/z* 431 and 434, respectively (N-O bond cleavage of the oxime), thus providing a useful diagnostic signals for aoWR derivatives.

P008
THE NCAM POLYSIALYLATION CAPACITY OF THE TWO POLYSIALYLTRANSFERASES ST8SIAII AND ST8SIAIV *IN VIVO*

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Introduction. The post-translational modification of the neural cell adhesion molecule (NCAM) with polysialic acid (polySia) plays an important role in the neuronal development of vertebrates. The presence of polySia is associated with the

expression of two key enzymes, the polysialyltransferases ST8SiaII and ST8SiaIV. In order to characterize the polysialylation competence of the two enzymes *in vivo*, we have analyzed NCAM of newborn mice differing in the number of ST8SiaII and ST8SiaIV alleles.
Experimental approach. PolySia chains in homogenized mouse brains were derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB) and separated by HPLC. The distribution of polySia chains in brain sections was analyzed immunohistochemically.
Results. The analyses revealed that each enzyme was individually able to synthesize polySia chains of more than 50 residues. Variations in the gene dosages of the enzymes resulted in alterations in the total amount of polySia and the chain length distribution.
Conclusions. Comparison of the degree of polymerization and the amount of polySia on NCAM strongly suggests a coordinated action of the two polysialyltransferases ST8SiaII and ST8SiaIV *in vivo*. In the perinatal phase, however, ST8SiaII appears to play the most important role.

P009
IDENTIFICATION AND CHARACTERIZATION OF KEYHOLE LIMPET HEMOCYANIN (KLH) N-GLYCANS MEDIATING CROSS-REACTIVITY WITH *SCHISTOSOMA MANSONI*

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Introduction. Keyhole limpet hemocyanin (KLH) of the mollusc *Megathura crenulata* is known to serologically cross-react with *Schistosoma mansoni* glycoconjugates in a carbohydrate-dependent manner. In order to elucidate the structural basis for this cross-reactivity, KLH N-glycans were analysed.
Experimental Procedures. Glycans were released from tryptic glycopeptides by PNGase F and fluorescently labeled. Cross-reacting glycans were identified using a polyclonal antiserum reacting with soluble *S. mansoni* egg antigens, isolated by a three-dimesional fractionation scheme and analyzed by different mass spectrometric techniques as well as linkage analysis and exoglycosidase treatment.
Results. The analyses revealed that cross-reacting species comprise about 4.5 % of released glycans. They all represent novel types of N-glycans with a Fuc(α1-3)GalNAc(ß1-4)[Fuc(α1-3)]GlcNAc-motif, which is known to occur also in schistosomal glycoconjugates. The tetrasaccharide unit is attached to the 3-linked antenna of a trimannosyl core, which can be further decorated by galactosyl residues, a xylose residue in 2-position of the central mannose and/or a fucose at the innermost *N*-acetylglucosamine.
Conclusions. Analysis of the carbohydrate structure of KLH proved to be an extremely difficult task due to the vast microheterogeneity of its glycans resulting in the expression of a great variety of different isomeric and/or isobaric structures. This study provides for the first time detailed structural data on the KLH carbohydrate entities responsible for cross-reactivity with glycoconjugates from *S. mansoni*.

P010
BIOTINYLATED CARBOHYDRATES FOR STUDYING LECTIN-CARBOHYDRATE INTERACTIONS

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Lectins are carbohydrate-binding proteins with a principle function in the recognition of carbohydrates present at cell surfaces, attached to circulating proteins, and present in extracellular matrices. By binding specifically to the carbohydrates, lectins mediate biological events, such as cell-cell adhesion, host-pathogen recognition, serum glycoprotein turnover, and innate immune responses.
Here we use biotinylated carbohydrates to study the binding specificity of plant and animal lectins. A simple and fast procedure for the biotinylation of carbohydrates based on reductive amination was developed. Labeling efficiency was determined by HPLC and mass spectrometry. Several biotinylated carbohydrates were immobilized on streptavidin-coated ELISA plates for binding assays with plant lectins. In a similar experiment, dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN), an adhesion receptor present as a cell-surface protein on dendritic cells that was shown to induce T cell proliferation, bound strongly to high-mannose and lewis b containing carbohydrates. We further showed that these two biotinylated glycans also bind to dendritic cells *in vitro*. Binding could be blocked by anti-DC-SIGN antibodies. Finally, DC-SIGN could be stained on lymph node tissue using biotinylated high-mannose *N*-glycan.
The data show that biotinylated carbohydrates can be used to determine carbohydrate-binding specificity of lectins that are expressed on cells, and can greatly improve the functional analysis of C-type lectins expressed on subsets of cells. The biotinylated carbohydrates can also be used to localize lectins on tissue, and therefore will be a great tool for investigating differential expression of C-type lectin receptors during disease development *in situ*.

P011
GLYCOMICS AND GLYCOPROTEOMICS

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Carbohydrates as constituents of glycoconjugates such as glycoproteins and glycolipids are increasingly being implicated in a wide diversity of biological and biomedical processes. However a major area of concern is that detailed structural knowledge of such carbohydrates are still limited. Our laboratory is addressing this problem by performing detailed mass spectrometric structural characterisation to produce glycosylation profiles of individual cell types, parasitic and free living nematodes, mice organs and human tissues. Additionally we are defining the glycans present on individual glycoproteins and sites of glycan occupancy by application of glycoproteomic strategies. Data from studies on *Schistosoma mansoni* glycoprotein secretions, glycosylation of a conserved domain in Murine ZP3 and transgenic Human ZP3 derived from mouse eggs, bacterial protein glycosylation and improved mass spectrometry techniques for the analysis of heparin oligomers will be presented.

P012
GANGLIOSIDE IMMUNOHISTOCHEMISTRY – SHOOTING AT A MOVING TARGET

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Numerous studies of ganglioside distribution have been published, but their results were somewhat conflicting and the situation is still not clear. Generally the inconsistencies in observed distribution were considered to be a consequence of different methods of fixation and varying specificity of antibodies, but in our hands even the same fixation procedure and highly specific monoclonal IgG antibodies were still producing inconsistent results. After numerous experiments we have realized that ganglioside immunohistochemistry is particularly sensitive to various influences. Even smallest amounts of organic solvents or detergents in any stage of the procedure (including fixation in cold solvents) will extract significant amounts of gangliosides from cell membranes and also enable their transfer from one cell, or tissue to another. For reproducible results all steps of ganglioside immunohistochemistry have to be performed at +4ºC, because at higher temperatures gangliosides shed significantly. Even when all steps are performed in cold and with utmost care, ganglioside immunohistochemistry still resembles shooting at moving targets, because gangliosides also jump from one cell or tissue to another in most of commercially available mounting media. In some of them this happens in a matter of hours, in some in a matter of days, but the picture is constantly changing. To make things even more complicated, different gangliosides jump to and from different structures, and some jumping can also be observed for GPI-linked proteins.

P013
FUNCTIONAL ANALYSIS OF THE CAMPYLOBACTER JEJUNI N-LINKED PROTEIN GLYCOSYLATION PATHWAY

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Recently an N-linked protein glycosylation system was identified in the human enteropathogen Campylobacter jejuni, the first example of this modification in a species from the domain Bacteria. We exploited the ability of this locus to function in E. coli to demonstrate through mutational and structural analyses that variant glycan structures can be transferred onto protein. Using glycoproteomic strategies exploiting ES-MS/MS technology, we describe the structural characterisation of N-linked glycans resulting from the mutation of individual genes from the C. jejuni N-linked glycosylation locus. Furthermore, structural data on these variant glycans allowed us to infer the role of individual glycosyltransferases in the biosynthesis of the heptasaccharide N-linked glycan. We also demonstrate that the putative oligosaccharyltransferase protein PglB, is able to transfer a variety of glycans onto C. jejuni proteins and also show that the C. jejuni and E. coli-derived pathways can interact in the biosynthesis of N-linked glycoproteins. These data provide a framework for understanding the process of N-linked glycosylation in Bacteria.

P014
ANALYSIS OF OLIGOSACCHARIDES RELEASED FROM GLYCOPROTEINS

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Introduction: Sample preparation and interpretation of mass spectrometric data has lagged behind technical development in glycan mass spectrometry, and has been a bottle neck for glycomic research. We have developed a seamless procedure

from sample preparation to a sugar structure report for analyzing glycans attached to glycoproteins.
Experimental: Glycoproteins were blotted onto PVDF membranes and oligosaccharides were released using PNGase F (N-linked) followed by reductive β-elimination (O-linked). Alditols were subjected to LC-MS and LC-MS/MS using a SGE ProteCol carbon column and Agilent Technologies' LC/MSD Trap XCT Plus. MS/MS data was automatically analysed using GlycomIQ software (Proteome Systems).
Results: Carbon chromatography provided excellent resolution for LC-MS of both N-linked and O-linked oligosaccharides, providing isomeric separation of common plasma oligosaccharides. Sample preparation (providing both released sialylated and desialylated oligosaccharides from glycoproteins) could be performed at sub-picomole scale providing excellent MS/MS. The software tools enabled the rapid prediction of possible sugar structures from the fragmentation data.
Conclusions: GlycomIQ together with the LC/MSD XCT ion trap provides high sensitivity robust automated MS/MS analysis for picomole to sub-picomole amounts of starting oligosaccharide. Automated data analysis and searching with state-of-the-art bioinformatics tools and database facilitates the analysis of glycoprotein biomarkers and recombinant glycoproteins.

P015
SITE SPECIFIC MAPPING OF POST TRANSLATIONAL MODIFICATIONS IN HUMAN ALPHA-1-ANTITRYPSIN

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Human alpha-1- antitrypsin (A1PI) is a well known glycoprotein in human plasma important for the protection of tissues from proteolytic enzymes. The three N-glycosylation sites of A1PI are endowed with diantennary N-glycans but also triantennary and unexpectedly even traces of tetraantennary structures leading to the typical isoelectric focussing pattern observed for A1PI which is due to different amounts of negative charges on the N-glycans. However, a detailed site specific structural mapping of the glycans of the A1PI isoforms has not yet been achieved. Here we present an approach to characterize A1PI from human plasma and its post translational modifications by a combination of LC ESI Q-TOF MS and MS/MS of the peptides obtained by proteolytic digestion. Up to 99% of the sequence including all glycosylation sites were covered. Variability with regard to the number of antennae and hence sialic acids was just detected on glycosylation site N107 whereas on N70 only minute amounts of triantennary structures were identified. Essentially only diantennary structures were present on site N271 in each of the isoforms analyzed. Fucosylation of diantennary structures was just marginal, whereas an increased fucosylation was detected for triantennary glycans. Exoglycosidase digests performed on the tryptic glycopeptides revealed that one of three neuraminic acids on triantennary structures was alpha 2,3 linked while all other sialic acids were alpha 2,6 linked.

P016
HIGH-RESOLUTION ELECTROSPRAY IONISATION MASS SPECTROMETRY IN STRUCTURAL STUDIES OF BACTERIAL LIPOPOLYSACCHARIDES

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Lipopolysaccharides of Gram-negative bacteria play an important role in the organization and function of the outer membrane and in the activation of the immune system of the host. Already small alterations in the chemical structure of the lipopolysaccharides may influence their biological activity. Due to intrinsic heterogeneity, structure elucidation of the lipopolysaccharides by chemical methods and NMR spectroscopy requires isolation of individual compounds, which is often complicated by a limited amount of the material available. Application of high-resolution MS may help to overcome this difficulty.
Electrospray ionisation Fourier transform ion-cyclotron resonance MS, including capillary skimmer dissociation and infrared multiphoton dissociation MS/MS techniques, was applied for studies of lipopolysaccharide-derived core oligosaccharide and lipid A samples as well as of the whole lipopolysaccharides of various bacteria. MS screening revealed 17 new monosaccharide sequences in the *Proteus* core oligosaccharides, whose general structure has been determined previously, and thus showed considerable lipopolysaccharide structure diversity in this genus. Data of fragmentation pathways revealed in the *Proteus* studies were used for elucidation of structures of the *Providencia* core oligosaccharides, which have not been studied earlier. Together with sugar analysis, this approach enabled identification of a common inner core, composition of the outer core and the nature of core heterogeneity, which is most commonly associated with different number of phosphate and ethanolamine phosphate groups. Studies of the intact rough-type lipopolysaccharide of *Yersinia pestis* helped to recognize structural details and temperature-dependent variations in both core and lipid A parts.

This work was supported by RFBR (project 05-04-48439).

P017
STUDIES ON THE FUCOIDAN CONSTITUENTS OF *CLADOSIPHON OKAMURANUS*

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Fucoidan refers to polysulfated polysaccharides commonly distributed in brown seaweeds composed mainly of fucose, sulfated fucose, galactose and glucuronic acid. The sulfated fucose components in fucoidan are believed to play an important role in exerting their beneficial bioactivities.

In the present study, we carried out acid hydrolysis under different conditions, optimized isolation procedures, and elucidated structures of fragment molecules. For separation, the hydrolysis products were labelled with a *p*-aminobenzoic ethyl ester (ABEE) chromophore and analyzed by HPLC and ESI-MS. Structural elucidation was carried out by ESI-MS and NMR spectrometry. As a result, we obtained two monosaccharides, two disaccharides, two trisaccharides and two tetrasaccharides : Fuc(1), Fuc-4-*O*-SO_3^-(2), GlcAβ1→2Fuc (3), Fuc-4-*O*-SO_3^--α1→3Fuc (4), GlcAβ1→2Fucα1→3Fuc (5), Fuc-4-*O*-SO_3^--α1→3[GlcAβ1→2]Fuc (6), Fuc-4-*O*-SO_3^--α1→3[GlcAβ1→2]Fucα1→3Fuc (7), Fuc-4-*O*-SO_3^--α1→3[GlcAβ1→2] Fuc-4-*O*-Acetyl-α1→3Fuc (8).
The ^{1}H-NMR spectra of ABEE labelled oligosaccharides displayed characteristic signals consistent with the presence of L-fucose, sulfated fucose, acetylated fucose and glucuronic acid unit. The sulfate ion appeared at *m/z* 96.0 in negative ion ESI-MS. The location of the sulfate group was confirmed by the ^{1}H and ^{13}C NMR signals characteristic to 4-*O*-sulfated fucose detected at δ_H 4.10-4.20 and δ_C 80-81.0 ppm. Further confirmation of the structure was made by DQF-COSY, HOHAHA and HMBC. To our knowledge, this is the first report on isolation and characterization of sulfated oligosaccharides from *Cladosiphon okamuranus* by MS and NMR. A search for biological activities of the hydrolysis products is under way.

P018
ADAPTATION OF THE "IN-GEL RELEASE METHOD" TO GLYCOME STUDIES FROM A VERY LITTLE AMOUNT OF MATERIAL

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N-glycosylation is a post-translational modification playing crucial physiological roles, for instance *via* cell-cell interactions. Notably for this reason, the N-glycan pattern varies depending on the organs, tissues and even the cell types. In some cases, such as in the studies on small organisms, obtaining enough starting material to allow the determination of the N-glycan present in a particular organ or tissue by conventional methods might be extremely complicated or indeed impossible.
A very sensitive technique, the "in-gel release method", allowing the determination of the N-glycans borne by one particular protein isolated by SDS-PAGE, has been developed, partially in our laboratory (Kolarich and Altmann 2000) during the last decade. Here we describe the adaptation of this method to obtain information on the glycome from a very small amount of tissue.
The starting material, a few milligrams of fresh tissue, is directly ground in 2-times concentrated SDS-PAGE buffer and electrophoresed for only a few minutes through a low percentage stacking gel. When the proteins start to separate on the denser resolution gel, the electrophoresis is stopped and the coomassie blue stained band containing the majority of the proteins is subject to the in-gel release method.
The developed technique was used to analyse the N-glycan pattern of several organs from plants and animals such as flowers of *Arabidopsis thaliana* and heads of *Drosophila melanogaster*.

P019
SYNTHESIS AND CHARACTERIZATION OF AN UNUSUAL GM2-DERIVATIVE, TAURINE-CONJUGATED GM2 FOUND IN TAY-SACHS BRAIN

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We have recently found that Tay-Sachs brain contains a taurine-conjugated GM2 (tauro-GM2), in which the carboxylic acid of Neu5Ac is amidated by taurine (*J. Biol. Chem.* 278, 35286, 2003). To understand the pathophysiological significance of tauro-GM2, we have synthesized this unique GM2-derivative and characterized its physico-chemical properties.
Chemical synthesis-The method involves the reaction of GM2 (223 mg, 0.16 mmol) with taurine (60 mg, 0.48 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride

(91 mg, 0.48 mmol), and 1-hydroxybenzotriazole hydrate (73 mg, 0.48 mmol) in 10 ml of dimethyl sulfoxide at 60°C for 30 min with stirring, followed by the addition of 1 ml of triethylamine. After stirring for an additional 1 hr at 60°C, the reaction mixture was desalted by using a Sep-Pak C_{18} cartridge. The product was purified by FPLC using a Mono Q 10/100 GL column. By this procedure, we obtained 139 mg of pure tauro-GM2 in 58% yield.
Physicochemical characterization- Conformational studies revealed that taurine conjugation on Neu5Ac increases the surface area occupied by the monomer inserted into a membrane and alters the dynamics of the oligosaccharide chain. In contrast to the Neu5Ac in GM2, a considerable flexibility is found for the tauro-Neu5Ac residue of tauro-GM2. This flexibility has also been confirmed by molecular modeling studies. Tauro-GM2 was converted to tauro-GM3 by β-hexosaminidase A and GM2 activator protein. However, tauro-GM3 was completely resistant to sialidases.

P020
ANALYSIS OF NONDERIVATIZED OLIGOSACCHARIDES BY CAPILLARY ELECTROPHORESIS-ELECTROSPRAY MASS SPECTROMETRY

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Post-translational modification of proteins through glycosylation can lead to heterogeneous mixtures of glycoforms. In this presentation, on-line capillary electrophoresis-electrospray mass spectrometry (CE-MS) was developed for the rapid, direct analysis of nonderivatized neutral and sialylated oligosaccharides.
A Prince CE system (Prince Technologies, The Netherlands) was coupled to a 4000 QTRAP mass spectrometer (Applied Biosystems/Sciex, Canada). Under the separation conditions, both neutral and sialylated oliosaccharides are detected in deprotonated forms (negative ion mode), protonated forms or as metal adducts (positive ion mode). In electrospray ionization, the formation of metal ion adducts are generally achieved by adding metal ions into the samples. However, it is well known that salts are not compatible with ESI because the presence of excess metal ions could suppress the ionization efficiency and leads to lower sensitivity. To overcome the problem, an on-line complexation approach has been developed to produce different metal ion adducts. With this approach, the fragmentation patterns of deprotonated ion, protonated ion, sodium and lithium adducts were investigated. As expected, a long- and short-range migration of a fucose was observed in protonated molecules, but not for the metal adducts. Even though protonated ions can lead to fucose migration, it was found that the protonated form provided structural information on sialylated oligosaccharide containing glycoforms. Therefore, we proposed that tandem mass spectrometry for both protonated ions and metal adducts should be performed in order to obtain sufficient information.

P021
SOLID PHASE OLIGOSACCHARIDE TAGGING (SPOT): A NEW AND EFFECTIVE METHOD FOR LABELLING OF REDUCING SUGARS.

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The emerging field of glycomics is daily being challenged by the lack of effective tools for high throughput characterisation of complex oligosaccharides. One problem associated with oligosaccharide analysis is the lack of proper spectroscopically detectable chromophores in reducing sugars and often it is necessary to label samples prior to analysis.
We have developed a new general labelling method termed SPOT that greatly speeds up the labelling and purification of reducing sugar from complex mixtures. The SPOT process is comprised of series of chemical manipulations that all have the potential of being fully automated as summarised below:

1) A sample containing reducing sugars is incubated with a solid support with a high loading of cleavable capture groups (hydroxylamines) that ensure complete capture of the sugars by formation of oximes.
2) Excess capture groups are blocked by treatment with a large excess of an acylating agent, like acetic anhydride.
3) The oxime double bond is selectively reduced with a borane-amine complex to give a reactive hydroxylamine.
4) A suitable tagging agent, preferably an aryl isothiocyanate like FITC or TRITC is added. Bromine or isotope containing tags for MS purposes may likewise be added.
5) Finally, the pure labelled sugar can be cleaved from the support and analysed.

The process has successfully been used for fluorescently labelling of monosaccharides (and mixtures thereof), linear and branched oligosaccharides including “real” samples released by PNG-ase digestion of glycoproteins. Isotope tags have also been added to demonstrate the method’s potential for differential glycomics using mass-spectrometry.

P022
ENZYME-ASSISTED SEQUENCING OF OLIGOSACCHARIDES ON BEADS USING SPOT (SOLID-PHASE OLIGOSACCHARIDE TAGGING)

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It is well known that glycosylation patterns on proteins and lipids strongly affect the biological role of these glycoconjugates. Structural analysis of oligosaccharides is therefore of major concern and still a considerable challenge for scientists.
A new technique - SPOT- has been developed in our laboratory where reducing carbohydrates are conveniently captured and fluorescently labeled on the solid-phase, and further cleaved into a small volume of concentrated product. In the present work, glycosidase treatment of such immobilized carbohydrates was investigated.
Tetramethylrhodamine (TMR)-labeled standards of the most common monosaccharide units found in eukaryotic glycoconjugates (D-Man, L-Fuc, D-Gal, D-Xyl, D-Glc, D-GalNAc, D-GlcNAc, and D-GlcA) were synthesized and conditions for their separation by capillary electrophoresis (CE) were optimized. The individual labeled monosaccharides could thus be identified by CE.
LNF I (Lacto-N-fucopentaose I: Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc) immobilized on hydroxylamine-functionalized controlled-pore glass (CPG)-beads was used as a model oligosaccharide and incubated with α-fucosidase and/or β-galactosidase in a sequence array-format experiment. Released Fuc and Gal residues could be captured either on the same beads (where digestion occurred) or on new beads, labeled with TMR and released by the SPOT technique. Monosaccharide analysis by CE, along with known standards, confirmed a terminal Fuc followed by a Gal residue.
We have here demonstrated that oligosaccharides immobilized on CPG by the SPOT technique are accessible to enzyme action and that the released carbohydrates can be further captured and labeled for analysis. This opens a promising approach for an easy sequence analysis of oligosaccharides.

P023
SYNTHESIS AND CHARACTERIZATION OF AN UNUSUAL GM2-DERIVATIVE, TAURINE-CONJUGATED GM2 FOUND IN TAY-SACHS BRAIN

Li, Y. (1); Ando, T. (1); Li, S. (1); Mauri, L. (2); Raimondi, L. (2); Bernardi, A. (2); Sonnino, S. (2)
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Chemical synthesis-The method involves the reaction of GM2 (223 mg, 0.16 mmol) with taurine (60 mg, 0.48 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (91 mg, 0.48 mmol), and 1-hydroxybenzotriazole hydrate (73 mg, 0.48 mmol) in 10 ml of dimethyl sulfoxide at 60°C for 30 min with stirring, followed by the addition of 1 ml of triethylamine. After stirring for an additional 1 hr at 60°C, the reaction mixture was desalted by using a Sep-Pak C_{18} cartridge. The product was purified by FPLC using a Mono Q 10/100 GL column. By this procedure, we obtained 139 mg of pure tauro-GM2 in 58% yield.
Physicochemical characterization- Conformational studies revealed that taurine conjugation on Neu5Ac increases the surface area occupied by the monomer inserted into a membrane and alters the dynamics of the oligosaccharide chain. In contrast to the Neu5Ac in GM2, a considerable flexibility is found for the tauro-Neu5Ac residue of tauro-GM2. This flexibility has also been confirmed by molecular modeling studies. Tauro-GM2 was converted to tauro-GM3 by β-hexosaminidase A and GM2 activator protein. However, tauro-GM3 was completely resistant to sialidases.

P024
***N*-ACETYLHEPAROSAN OLIGOSACCHARIDES: MICROSCALE PREPARATION AND MS/MS FRAGMENTATION ANALYSIS**

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N-Acetylheparosan (NAH) is a glycosaminoglycan known as a biosynthetic precursor of heparin and heparan sulfate (Hep/HS). Its structure is composed of simply alternating α1-4 linked GlcNAc and β1-4 linked GlcA, and is not sulfated at all. Enzymatic digestion of NAH and subsequent chromatographic purification steps can afford size-defined NAH oligosaccharides, which should be valuable not only as research tools for Hep/HS-relating enzymes, but also as modification substrates for syntheses of a wide variety of derivatives.
In this study, K5 polysaccharide produced by *Escherichia coli* strain K5 were partially depolymerized with heparitinase I into a mixture of even-numbered NAH oligosaccharides, having an unsaturated uronic acid at the non-reducing end. Furthermore, a mixture of odd-numbered oligosaccharides was derived by removing the unsaturated uronic acids in the above mixture by a reaction with mercury (II) acetate. Each of the oligosaccharide mixtures was subjected to gel-filtration chromatography to give a series of size-uniformed NAH oligosaccharides with satisfactory purity (assessed by analytical anion-exchange HPLC), and their oligosaccharide lengths were determined by MALDI-TOF-MS and ESI-IT-MS.
MALDI-TOF/TOF-MS/MS and ESI-IT-MS^n spectra of the

purified NAH oligosaccharides were acquired and examined in detail. The fragmentation patterns observed were of high regularity with characteristic glycosidic cleavages, reflecting the ruled structure of NAH, but were distinct from those obtained for hyaluronan oligosaccharides. The data obtained here should be extremely valuable for their sensitive identification and as fundamentals for future analyses of modified NAH oligosaccharides, i.e., Hep/HS-relating oligosachharides.

P025

CAPILLARY ELECTROPHORESIS-ELECTROSPRAY MASS SPECTROMETRY FOR RAPID AND SENSITIVE ANALYSIS OF *N*-GLYCANS AS 9-FLUORENYLMETHYL DERIVATIVES

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Most proteins show characteristic biological roles after modification with glycans. Comprehensive analyses of glycans are important for understanding the protein function. In contrast to proteomics, high-throughput glycomics analysis is still in developing stage. In this study, glycosyl amine forms of the reducing end of glycans released from glycoprotein with peptide:*N*-glycanase F (PNGase F) were directly derivatized with 9-fluorenylmethyl chloroformate (Fmoc-Cl), and analyzed by capillary electrophoresis-electrospray ionization mass spectrometry (CE-ESI MS).
Procedure: Glycoprotein samples (10 μg) were digested with PNGase F (1 unit) in phosphate buffer (pH 8.5). The mixture was incubated at 37^{o}C for 2 h. Fmoc-Cl in acetone (200 μL, 50 mg/mL) was added to the mixture, and the mixture was incubated at 37^{o}C for 1 h. After removing the excess reagent and protein by extraction of the mixture with chloroform, a portion of the aqueous layer was analyzed by CE-ESI MS.
CE-ESI MS and CE-ESI MS/MS using Fmoc derivatives are a powerful technique for the structure analysis of glycans derived from high-mannose, sialo- and asialo- complex and hybrid-type oligosaccharides. We applied the method to the analysis of glycans derived from some antibody pharmaceuticals, and found that the pharmaceutical products from different cells showed characteristic patterns of carbohydrates. We will also show that Fmoc-CE-ESI-MS technique is useful for identification of oligosaccharides released from the band observed on 2-DE gel after in-gel digestion. The method requires only 4 hours to perform the whole procedures including the release of glycans, derivatization with Fmoc and analysis by CE-ESI MS.

P026

CHARACTERIZATION OF N- AND O-GLYCANS DERIVED FROM PLASMA-DERIVED AND RECOMBIANANT FACTOR VII

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Highly efficient separation techniques, laser-induced fluorescence (LIF) detection, and different mass-spectrometric (MS) measurements were combined in a multi-methodological model to perform a comprehensive structural characterization of N- and O-linked oligosaccharides found in plasma-derived and recombinant factor VII. As a first approach, peptide mapping (trypsin and AspN), using liquid chromatography coupled to a quadrupole/time-of-flight, was used to determine O-glycan structures within Ser52 and 60 and N-glycans on Asn145 and 322. Then, oligosaccharide mapping was carried out both through capillary electrophoresis (CE)-LIF and normal phase liquid chromatography (NP-HPLC) methods, in which the enzymatically released N-glycans were fluorescently labelled. These analyses combined with matrix-assisted laser desorption/ionization (MALDI)-MS profiling of the intact glycan structures provide full assessment of the different structures. Linkages and monosaccharide residues were confirmed by successive exoglycosidase digestions. Thus, MALDI-MS, CE-LIF, and NP-HPLC data were effectively combined to reveal the overall structural diversity and to quantify both acidic and neutral glycans. These complementary methodologies were successfully applied to highlight glycosylation differences within both plasma-derived and recombinant factor VII.

P027

DIRECT NANOELECTROSPRAY IONIZATION MASS SPECTROMETRY ANALYSIS OF GLYCOPEPTIDES DERIVED FROM IN-CAPILLARY OR IN-SOLUTION PROTEOLYTIC DIGESTS OF GLYCOPROTEINS

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We recently developed a method for direct identification and/or sequencing of proteins by proteolytic digest in the electrospray capillary and simultaneous analysis of the resulting peptides by nanoESI MS and MS/MS [1]. This strategy was now applied to the analysis of glycoproteins.
Glycoproteins were dissolved in 10 mM ammonium hydrogen carbonate containing 10% methanol. After adding the protease

(trypsin or chymotrypsin) the mixture was transferred to the electrospray capillary and data acquisition was started.
When ribonuclease B was subjected to tryptic degradation in the electrospray capillary a number of glycopeptides could be detected. They were identified by CID experiments as $_{60}NLTK_{63}$ attached to $Man_{4-8}GlcNAc_2$ and $_{58}SRNLTK_{63}$ linked to $Man_{4-5}GlcNAc_2$ residues.
In-capillary tryptic digest of asialofetuin revealed N- and O-glycopeptides. Oligosaccharide and peptide fragments derived from CID experiments allowed the determination of the glycan structures as well as of the peptide sequences. The N-glycans of all three glycosylation sites could be identified as triantennary complex type structures. One of the O-glycosylation sites was identified as T_{280}, since the corresponding glycosylated peptide fragment ions were detected in the CID spectrum.
Since the sialo-N-glycans of transferrin do not efficiently ionize under in-capillary digestion conditions, transferrin was degraded in-solution by use of chymotrypsin and trypsin subsequent to a DTT treatment. Addition of methanol and formic acid allowed direct analysis of the digestion mixture. Again CID provided information on the glycan structure (diantennary, disialylated) and the aa sequence of the glycopeptides.

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P028
IMMOBILIZATION OF 2-AMINOPYRIDINE-OLIGOSACCHARIDES: BRIDGING STRUCTURAL ANALYSIS AND GLYCOARRAYS

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Printed carbohydrate micro array reported recently [1] requires nanomolar quantities of carbohydrate ligands. On the other hand, routine structure analysis of glycoprotein carbohydrate chains based on chromatographic separation of oligosaccharides is also often performed within nmol range. This coincidence opens an attractive prospect of using as obtained after *analytical* HPLC for immobilization on chip followed by assaying lectins and other carbohydrate-binding proteins. Such a "link" variant when as bears a fluorescent label is especially attractive: on the one hand, this makes easier the HPLC separation and more sensitive as detection, on the other hand, makes real quantitative dosage of glycans during chip fabrication. In this case the experimental technique could be the following: i) carbohydrate chains are cleaved from protein core, *ii)* oligosaccharide pool is labeled with fluorescent reagent, *iii)* HPLC is performed, possibly in two-dimensional mode, i.e. using consequently two columns, ivY after detection the collected peaks are diluted or concentrated to the optimal concentration, and v) are placed to microfluidistic system of printing robot.
In this work, we demonstrated the possibility of grafting onto chip the widely used and commercially available, 2-aminopyridine (2AP) derivatives of oligosaccharides. The chip is 3D gel microchip developed earlier for oligonucleotide assaying [2].

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P029
DEVELOPMENT OF A VERSATILE KIT FOR HIGH-THROUGHPUT OLIGOSACCHARIDE PURIFICATION VIA CHEMOSELECTIVE GLYCOBLOTTING

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The difficulty of oligosaccharide pattern analysis of glycoconjugates mainly depends on the complicated procedure of oligosaccharide purification. Previously we reported that rapid and efficient purification was achieved using an oxylamine-containing material on the principle of chemoselective conjugation[1, 2]. The chemoselective ligation of oligosaccharides in a crude mixture onto the materials relies on the oxime bond formation between oxylamine present on the material and aldehyde or ketone group present at the reducing terminal of oligosaccharide, thus enabling highly selective and high-throughput oligosaccharide purification. Oligosaccharides conjugated onto the materials were recovered as intact form by acid hydrolysis or reduction, which might limit the high sensitive detection. Aiming to improve the detection sensitivity, we applied a novel strategy to label chemoselectively-trapped oligosaccharide by oxime exchange reaction. We found trapped oligosaccharide on the material could be effectively released under mild condition by adding large excess *O*-substituted oxylamines in a neutral or weakly acidic condition. The released oligosaccharides were recovered as oxime of *O*-substituted oxylamine added, thus enabling simultaneous releasing and derivatization. The same strategy was applied to the purification of glycopeptides that was previously treated with galactose oxidase to introduce aldehyde groups at the nonreducing end's galactose residue. Furthermore, we improved the base polymer matrix by synthesizing a novel oxylamine containing monomer, which realized high stability under harsh washing conditions, high capacity, and extremely easy handling protocols. The optimized protocol was successfully applied for the glycomic

analysis of human serum. These technologies were commercialized as an oligosaccharide purification kit 'BlotGlyco™'.

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P030
HIGH THROUGHPUT QUANTITATIVE GLYCOMICS AND GLYCOFORM-FOCUSED PROTEOMICS OF MURINE DERMIS AND EPIDERMIS

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Introduction Recent progress in mass spectrometry demonstrated their ability to provide information both on peptide sequence and glycan structure for the analysis of glycopeptide. However, their throughput is not high enough to apply large scale protein glycosylation analyses. Therefore, mining the significance of protein glycosylation requires further strategy. One solution is to develop a focused approach based on function and information content.
Experimental Gross *N*-glycan profiling of murine epidermis and dermis were elucidated both qualitatively and quantitatively by novel stable isotope coded glycan-selective derivatization reagents, (aoWR(H) and aoWR(D)). Glycoproteins carrying unique oligosaccharides were then selectively analyzed following direct tryptic digestion of protein mixtures, affinity enrichment and off-line LC-MALDI-TOF/TOF.
Results The novel derivatization reagents allowed a rapid and sensitive *N*-glycan profiling on MALDI-TOF in a quantitative manner both intra- and inter-samples. The glycomic analysis revealed distinct features of *N*-glycosylation profile of epidermis and dermis. A feature worthy of mention is that the murine epidermis glycoproteins are characteristic in their high abundance of high-mannose type oligosaccharides. Based on this observation, we performed focused proteomics that carry high-mannose type glycans. We identified 15 glycoproteins with 19 *N*-glycosylation sites by off-line LC-MALDI-TOF/TOF mass spectrometry. Moreover, the relative quantitation of microheterogeneity of different glycoforms present at each *N*-glycan binding site was determined. Glycoproteins identified were mainly content of lysosomes, lamellar granules and desmosomes.
Conclusion This study demonstrates the gross *N*-glycan profiling prior to glycoprotein identification makes the particular glycoforms-focused approach functionally meaningful and greatly accelerate the data mining process.

P031
CHARACTERISATION OF GLYCOSYLATION ON RABBIT ERYTHROCYTES

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Murine sperm initiate fertilisation by binding to the specialised extracellular matrix of their homologous eggs known as the zona pellucida (mZP). There is substantial controversy about the molecular basis of this interaction although one of the prevailing views is that it is carbohydrate dependent. Surprisingly, murine sperm also bind very tightly and with high specificity to rabbit erythrocytes. Earlier studies indicate that this somatic cell adhesion system is also carbohydrate dependent. All of the N- and O-linked glycans associated with the mZP have now been characterised. Accordingly, an important objective is to establish any glycosylation overlap between rabbit erythrocytes and mZP. Using ultra-high sensitivity MALDI-TOF, MALDI TOF/TOF and GC mass spectrometry in combination with exoglycosidase digestions we have characterised the N- and O-glycans on rabbit erythrocytes. Our data suggest that rabbit erythrocytes are abundantly N-glycosylated with highly branched LacNAc N-glycans but are remarkably deficient in O-glycosylation. Notably, the O-glycans that are expressed on both rabbit erythrocytes and mZP have not been previously implicated in initial murine sperm-egg binding. Interestingly, a substantial subset of the N-glycans associated with rabbit erythrocyte membranes are structurally very similar to those expressed on mZP3. These results suggest the possibility that N-glycans could play a central role in mediating robust sperm binding during both fertilisation and in this very unusual somatic cell adhesion system. These studies demonstrate that this powerful technology is ideally suited for probing complex glycoylation heterogeneity in cell adhesion systems.

P032
SYNTHESIS OF CHONDROITIN SULFATE E OLIGOSACCHARIDES VIA AN EFFECTIVE GLYCOSYLATION

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Chondroitin sulfates, one of the members of glycosaminoglycan family, have been revealed to have the important biological properties. Especially, "chondroitin sulfate E" that is composed of highly sulfated disaccharide unit, β-D-GalNAc(4,6-di-O-SO_3Na)-(1→4)-β-D-GlcA, binds many types of specific biological molecules such as midkine (1), type V collagen (2), L-, P-selectin and chemokine (3) at poly- or oligosaccharide level. However, we have little information about the optimum structures including the sequence, length and the aglycon of the glycan chain which might affect the biological activities. In order to understand these characteristics in more detail at the molecular level, structurally defined oligosaccharides of chondroitin sulfate E are strongly required. Here we report the synthesis of chondroitin sulfate E oligosaccharides in the repeating region possessing GlcA residue at the reducing terminal. We performed the glycan elongation in high yields by employing a suitable disaccharide unit, β-D-GalNAc-(1→4)-β-D-GlcA. Successful glycosylations of di- + di-, mono- + tetra-, and di- + tetrasaccharide were rationalized by the formation of the reactive intermediates deeply concerned with the acyl protecting group and the acetamide at the glycosyl donor and acceptor, respectively. We thus obtained the targeted chondroitin sulfate E penta- and hexasaccharides in an efficient way.

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P033
COMBINATION OF ESI-Q-TOF AND MALDI-TOF FOR THE ANALYSIS OF HEPARIN OLIGOMERS

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Heparin and heparan sulfate (HS) are complex, N- and O-sulphated and N-acetylated polymers of alternating glucosamine and hexuronic acid. They have several important biological activities mediated by specific interactions with targeted proteins. The concept of specificity relies upon the existence of specific domains within the heparin/HS chain. Despite being of extreme importance, their structural characterization remains difficult because of the variety of the substitution patterns as well as the general low abundance of these products. Mass spectrometry is one of the most powerful techniques for heparin/HS structural characterization and among the mass spectrometry methodologies, electrospray-ionization (ESI) has been most successful; however the high charges of the heavily sulphated oligomers compromise discrimination between components with similar masses. Matrix Assisted Laser Desorption Ionization (MALDI) is capable of sufficient mass precision but MALDI analysis of native HS samples has been rarely pursued because of the difficulty of ionizing such highly-charged molecules.
We have used different mixes of matrices using mainly norharmane to successfully analyse heparin oligomers (produced by partial heparinase digestion) by MALDI-MS. Although the species observed by MALDI are highly de-sulphated useful structural information can be deduced. Moreover we observe in MALDI a minor component which corresponds to the same-DP-chain where 1 sulphate group has been exchanged against 2 N-acetyl groups (mass difference of 4). This species could not be unambiguously identified by ESI alone. Thanks to the combination of MALDI and ESI results we have been able to identify all the major components in heparin-oligomer mixtures from DP4 to DP8.

P034
ANALYSIS OF FLUOROPHORE LABELED GLYCOSAMINOGLYCAN DISACCHARIDES USING POLYACRYLAMIDE GEL ELECTROPHORESIS AND HPLC

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Glycosaminoglycans (GAGs) play pivotal roles in many cellular events and in the biomechanical properties of a wide variety of tissues. The knowledge of the nature and relative amounts of these GAGs is necessary for the understanding of their role in both healthy and pathologic tissues.
The aim of this work was to develop a PAGEFS (polyacrylamide gel electrophoresis of fluorophore labeled saccharides), a method suitable for the analysis of HA, DS/CS and also HE/HS derived Δ-disaccharides. PAGEFS relies on derivatization of reducing end of Δ-disaccharides with 2-aminoacridone (AMAC) followed by electrophoresis under optimised conditions in polyacrylamide gels and buffers. Results showed that the choice of the electrophoresis buffer, consisting of Tris-HCl and Tris-Borate, pH and the concentration of cross-linkers (N,N'-methylenebisacrylamide) in the gels were critical for efficient separation. Qualitative and quantitative analysis of the bands was performed using a sensitive imaging system. The sensitivity of the developed PAGEFS methods was found to be 25pmol per disaccharide.
To confirm these data, analysis of the same standards of GAG disaccharides, labeled with AMAC, were analyzed using the HPLC technique, using a reverse phase column (C18) and a

fluorophore detector, with λem= 442 and λex= 520 for the identification of the AMAC-disaccharides.
These methods were applied to the medium of cell cultures and varius tissues. Specifically, GAGs were isolated and then degraded using either hyaluronidase SD and chondroitinase ABC, for the HA and DS/CS isolation, or a mix of heparanases I, II and III, for the HE/HS isolation, followed by AMAC-derivatization.

P035
BETA-LACTOGLOBULIN GLYCOCONJUGATES : IMPACT OF THE DEGREE OF POLYMERIZATION OF THE SUGAR ON THE MAILLARD REACTION KINETICS

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Among the different milk proteins, whey proteins are well known for their high nutritional value. They are used as functional ingredients in a wide variety of dairy products such as ice cream, yoghurt etc. An estimate of the worldwide production amounts to approximately 700 thousand tonnes available as valuable food ingredient [1]. β-lactoglobulin (BLG) is the predominant protein in whey protein isolates (>50 %). It is commonly used to stabilise food emulsions because of its surface-active properties.
It is well known that modification of food proteins by covalent bonding of sugars markedly improve their functional properties (solubility, heat and pH stability, emulsifying ability, gel forming properties etc) [2] as well as decrease allerginicity.
First stage Maillard reactions (condensation of reducing sugars with amino groups i.e. mainly lysine residues) could be performed either in a dry state or in aqueous solution. The dry state glycation does not alter significantly the native structure of the protein [3] and has been demonstrated to be more efficient [4].
Whereas monosaccharides have been extensively studied, the conjugation of oligo- and polysaccharides to BLG remains widely unknown. In this study, malto-oligosaccharides have been prepared by acidic hydrolysis and separated in pure form depending on their degree of polymerisation (controlled DP<10) by preparative HPLC. Then, they have been grafted through dry-glycation Maillard reaction onto BLG. The glycation behaviour has been assessed through kinetic experiments and followed by mass spectrometry (LC/ESI-MS).

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P036
***GENDER-SPECIFIC EXPRESSION OF N-GLYCANS BY THE BLOOD-FLUKE* SCHISTOSOMA MANSONI**

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Schistosoma mansoni are human parasites, widely occurring in tropical regions, that live as adult male/female egg-laying couples in the blood stream of the host. We compared the PNGase F-released N-glycan populations from glycoproteins of male and female schistosomes by MALDI-TOF-MS and nano-RP-LC-MS/MS after labelling with 2-aminobenzamide. N-glycans with LacNAc-antennae were found to be abundantly present in females, but where hardly present in males. These N-glycans comprised partially truncated diantennary and triantennary core-fucosylated structures with up to three additional fucose residues on the antennae forming terminal Lewis X elements. On the other hand, N-glycans of males were dominated by LacdiNAc (LDN)-containing structures, which were of significantly lower abundance in females. Notably, dimers of LDN were observed, which have sofar not been reported from natural sources, but have recently been obtained by the expression of a *C. elegans* GalNAc transferase in Chinese hamster ovary Lec8 cells (Kawar et al., J. Biol Chem (2005) 13:12810). LDN- and diLDN-containing N-glycans of *S. mansoni* males comprised monoantennary and diantennary species which were core-fucosylated and contained additional fucose residues on the antennae, resulting in GalNAcβ1-4(Fucα1-3)GlcNAcβ1- (LDNF) as well as di-LDNF motifs. Furthermore, male and female flukes expressed comparable relative amounts of oligomannosidic N-glycans.
In conclusion, partly fucosylated LacNAc antennae are preferentially expressed by female schistosomes, while partly fucosylated LDN and diLDN antennae are predominantly found on N-glycans of male schistosomes. This sex-specific glycosylation may play a role in the recognition/communication of the male and female flukes and might have further implications in the host-parasite interaction.

P037
ANALYSIS OF SULFATED SIALIC ACIDS IN SEA URCHIN SPERM AND EGG

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Sialic acids (Sia) consist of more than 50 derivatives that are mostly modified by various substitutions such as acetylation, methylation and sulfation. Sulfated Sia are known to occur in glycolipids and glycoproteins of sea urchin gametic cells and in gangliosides of bovine stomach. However, little is known about the occurrence in other animal cells and tissues, because of limitations of analytical methods for detecting minute amounts of sulfated Sia. Therefore, we began to develop methods for detecting the sulfated Sia, and here we show chemical and immunochemical methods that were applicable to sea urchin gametic cells. (i) Fluorometric HPLC analysis: Samples were hydrolyzed under acidic conditions and the hydrolysates were derivatized with DMB (1,2-diamino-4,5-methylenedioxybenzene), followed by HPLC analysis. 8-*O*- and 9-*O*-sulfated forms of Neu5Ac, Neu5Gc and KDN could be separated on the HPLC. (ii) Immunochamical methods using anti-sulfated Sia monoclonal antibodies: For generation of monoclonal antibodies, mice were immunized with glycoconjugates from sea urchin gametic cells. Three monoclonal antibodies were obtained: mAb.3G9, specific to 8-*O*-sulfated Neu5Ac (Neu5Ac8S); mAb.2C4, specific to Neu5Ac/Gc8S, and mAb.H4C2, specific to Neu5Ac/Gc8Sα2,8Neu5Ac/Gc. We applied these methods and antibodies to glycoproteins and glycolipids from sea urchin sperm and egg. It was demonstrated that sperm glycoconjugates exclusively contained Neu5Ac8S, while egg glycoconjugates contained Neu5Gc8S. We further analyzed the content of Neu5Gc8S in eggs from two different sea urchin species, and showed that the Neu5Gc8S residue was localized not only in the egg surface, but also in the egg cytoplasm.

P038
DETECTION OF ENDOTOXIN IN RAT SERUM USING 2-KETO-3-DEOXYOCTULOSONIC ACID (KDO) AS CHEMICAL MARKER DETERMINED BY GAS-LIQUID CHROMATOGRAPHY - TANDEM MASS SPECTROMETRY (GLC/MSMS)

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Lipopolysaccharides (LPS, endotoxins) are major components of the outer membrane of Gram-negative bacterial cell envelope. LPS possess immunostimulatory and toxic properties to human organism even in trace amounts. Detection and quantitation of endotoxins in biological fluids, environment or biotechnological products is a matter of great importance for medicine as well as for the industry.
In the present work a gas-liquid chromatography – tandem mass spectrometry (GLC-MSMS) was applied to the detection of 3-deoxy-D-*manno*-2-octulosonic acid (Kdo), a constituent of bacterial endotoxin (LPS), in rat serum samples. Two groups of rats were treated with intraperitoneal injections of LPS from *E. coli* O127:B8 (Sigma) dissolved in pyrogen-free 0.9% NaCl solution. The first group received a single LPS dose of 20mg/kg of body weight, second group received LPS injections of 2mg/kg over ten days (total dose of 20mg/kg body weight). Kdo level in the serum samples was measured in comparison to the third group of serum samples from animals treated with intraperitoneal injection of saline only. Samples were dried, methanolyzed, acetylated and analyzed by GLC-MSMS. The level of Kdo per ml of serum in the fist group was on average 10 times higher than in the second group and 60 times higher than in control group. Endotoxin level in the samples was tested also with Limulus Amebocyte Lysate (LAL) test. Comparison of results obtained by both methods indicate, that GLC-MSMS provides information about the total LPS content, whereas LAL test detects only part of endotoxin present in the sample.
Acknowledgements: The research was supported by the grant No. 3 P04A 014 23 of the Ministry of Science and Information Society Technologies in Warsaw, Poland

Synthesis of glycoconjugates and glycomimetics

P039
INCREASE IN THE GROWTH INHIBITION OF BOVINE PULMONARY ARTERY SMOOTH MUSCLE CELLS BY AN *O*-HEXANOYL LOW MOLECUALR WEIGHT HEPARIN DERIVATIVE

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Heparin's (HP'S) antiproliferative effect on bovine pulmonary smooth muscle cells (BPASMCs) is potentially important in defining new approaches to treat pulmonary hypertension. The proliferation of pulmonary artery smooth muscle cells (PASMCs) appears to play a significant role in pulmonary hypertension. Commercially available HPs vary widely in their antiproliferation properties. In order to enhance antiproliferative potency of HP , we have prepared *O*-hexanoylated HP in two steps, as follows: (a) HP was treated by periodate followed by sodium borohydride and (b) the tributylammonium salt of this HP derivative was *O*-acylated with hexanoic anhydride. Gradient polyacrylamide gel

electrophoresis showed that the major heparin fragment contained 8 disaccharide units. NMR analysis showed that approximately 1 hexanoyl goup per disaccharide residue was present. The *O*-hexanoylated heparin fragment was assayed for the growth inhibitory effect on bovine PASMCs in culture. This HP derivative is found to be more effective in the growth inhibition of bovine PASMCs in culture than the heparin from which it was derived. In the future, it is envisioned that this or similar derivatives may be an effective treatment for pulmonary hypertension. Supported by NIH Grants # HL39150 and HL52622.

P040
SYNTHESIS OF A LINEAR MANNOPENTAOSIDE, A REPEATING UNIT OF LIPOPOLYSACCHARIDES FROM *ESCHERICHIA COLI* O9 AND *KLEBSIELLA PNEUMONIAE* O3, AS 2-CARBOXYETHYLTHIO GLYCOSIDE

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O-Specific chains of lipopolysaccharides from *Escherichia coli* O9 and *Klebsiella* pneumoniae O3 with high adjuvant activities are built of a pentasaccharide repeating unit [1, 2]
-3)-α-D-Man*p*-(1-3)-α-D-Man*p*-(1-2)-α-D-Man*p*-(1-2)-α-D-Man*p*-(1-2)-α-D-Man*p*(1-
Here we present an approach to the synthesis of a pentasaccharide fragment of the polysaccharide as 2-carboxyethylthio glycoside according to a [2+3] scheme. (1-3)-Linked mannobiosyl trichloroacetimidate **1** served as a glycosyl donor and (1-2)-linked trisaccharide **2**, as a glycosyl acceptor. The pentasaccharide with a 2- carboxyethylthio aglycon will be used in the studies of adjuvant activity.

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P041
COMPREHENSIVE STUDIES ON THE REGIOSELECTIVITY IN THE TRANSGLYCOSYLATION USING β-GALACTOSIDASES OF VARIOUS ORIGINS.

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We have shown that a recombinant β-galactosidase from *Bacillus circulans* ATCC 31382 gives β-1,3-linked disaccharide exclusively in the transglycosylation reaction. However, it was revealed that the regioselectivity was low when Gal[1)] or Man[2)] was used as an acceptor. For the purpose to solve this discrepancy, we compared the regioselectivities of the transglycosylation to Gal or Man using six β-galactosidases from various origins. In that case too, most enzymes showed low-regioselectivity in the reaction to Gal or Man as acceptor.[3)]
In the present study, we summarily re-investigated the regioselectivity in the transglycosylation using various β-galactosidases. As enzyme sources, we selected β-galactosidases from *E. coli*, *Aspergillus oryzae*, *Penicillium multicolor*, *Bifidobacterium bifidum*, *Bacillus circulans,* and above recombinant enzyme. We examined the regioselectivity of these enzymes in the transglycosylation using *para*-nitrophenyl-β-D-galactopyranoside (Gal-β-pNP) as a donor, and Glc, Gal, Man, GlcNAc, and GalNAc as acceptors.
Only the reaction using GlcNAc or GalNAc as acceptor, the reaction resulted in the well known high regioselectivity. In contrast, the reaction using Gal or Man as acceptor, the regioselectivity was disordered, and the reaction using Glc showed intermediate regioselectivity. Therefore, the so-called regioselectivity, in conclusion, can be said as a regioselectivity only in the reaction using GlcNAc or GalNAc as acceptor, and a caution must be paid when using Glc, Gal, or Man as acceptor in every β-galactosidases.

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P041b
NEOGLYCOCONJUGATES: STEREOSELECTIVE SYNTHESIS OF α-GLYCOSYL AMIDES

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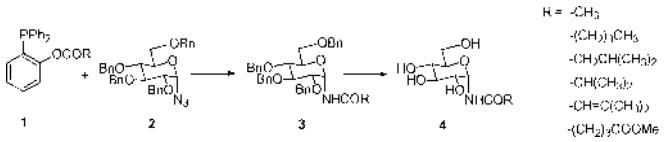
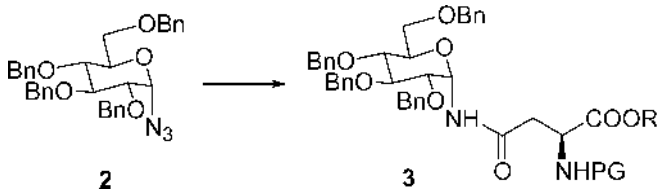
It has been recently shown that the conformation of the peptide in α- and β-*N*-linked glycopeptides is uniquely influenced by the attached sugar in ways that depend on the configuration of the carbohydrate-peptide linkage.[1] The stereoselective synthesis of neo-glycoconjugates in the "unnatural" α configuration has therefore become of great interest as a means of designing glycopeptide mimetics which can be useful as probes or inhibitors of glycosyltransferases.
We have recently reported[2] the first general method for the reductive acylation of α-glycosyl azides that proceeds with retention of configuration at the anomeric carbon using the functionalised phosphines **1**.[3] The reaction could be applied to *O*-benzyl glycosyl azides in the fuco-, gluco- and galacto-

series with good yield and selectivity. Removal of the protecting groups yielded the α-glycosyl amides **4**.



The mechanism of the reaction was studied, and some of the intermediates were isolated and identified by NMR spectroscopy. The 3D structure of the □-glycosyl amides was also investigated by NMR spectroscopy, revealing that the pyranose ring maintains the $^4C_1$ conformation.



The course of the reaction and the stereoselectivity appear to be influenced by many factors, most notably the structure of the acyl group to be transferred and the nature of the solvent.
N-glycosylation of aspartic acid side chain is particularly useful to obtain neo-glycopeptides.
For this purpose the *o*-diphenylphosphinophenol was acylated with variously protected L-aspartic acid The corresponding acyl phosphines were used for the reduction-acylation of □-glucosyl azide, yields and selectivity varying with the reaction conditions (solvent and temperature).

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P042
ANTI-TUMOR-PROMOTING ACTIVITY OF NEW GALACTOGLYCEROLIPIDS ANALOGUES AND THEIR EFFECT ON TPA INDUCED PKC EXPRESSION

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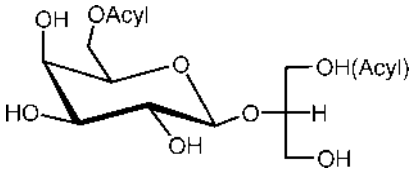
INTRODUCTION Chemoprevention is aimed to identifying agents with potential preventive roles in cancer [1]. Several 2-O-glycosylglycerols with a hexanoyl group in position 1 of the glycerol, or 6 of the sugar, have shown inhibitory effect on tumor promotion [2]. As the acyl chain plays an important role in modulating the biological activity of these compounds, the synthesis of new galactoglycerolipid analogues was planned, in which one or two branched, alicyclic or aromatic acyl chains are linked to the 6'- or 1 and 6'-position of 2-O-β-D-galactosylglycerol. The new compounds were tested for in vitro anti-tumor-promoting activity and the effects on TPA-induced PKC expression was evaluated for two of them.
METHODS The glycoglycerolipid analogues were prepared through chemoenzymatic procedures in which the acyl chains were linked to the 6' and 1 positions of 2-O-β-D-galactosylglycerol by lipase catalyzed transesterification, or by acylation of a temporarily protected galactosylglycerol intermediate. The anti-tumor-promoting activity of the prepared compounds was evaluated basing on the short term in vitro assay for the inhibition of Epstein-Barr virus early antigen (EBV-EA) activation induced by the tumor promoter TPA, and the effects on TPA-induced PKC expression on mouse skin were studied through western blot analysis.
RESULTS Fifteen new compounds were prepared. They were active in inhibiting the EBV activation promoted by TPA, the branched analogues resulting the most active of the series. Western blot analysis showed down-regulation or up-regulation for TPA-induced PKC expression when a branched or a alicyclic derivative was applied respectively on mouse skin.



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P043
ENZYMATIC SYNTHESIS OF Tn ANTIGEN, A TUMOUR- ASSOCIATED CARBOHYDRATE ANTIGEN

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Tn antigen (2-deoxy-2-acetamido-α-D-galactopyranosyl-*O*-serine, GalNAc-α-*O*-Ser) is a tumour-associated carbohydrate antigen found in many tumour types. The development of efficient synthetic strategies for this compound is essential both for diagnostics and therapeutic purposes [1-3].
In this work, a comparison between different strategies aimed at the enzymatic synthesis of Tn antigen is reported. The reactions were performed using 4-Nitrophenyl *N*-acetyl-α-D-galactosaminide (Gal*N*Ac*p*NP) as the donor and α-*N*-Acetyl-galactosaminidase from *Acremonium sp.* as the synthesis promoter.
Several advanced analytical techniques have been used for the assessment of kinetics in different reaction conditions, including capillary electrophoresis with UV detection and high-performance liquid chromatography with electrospray mass spectrometry detection. The optimization of reaction conditions, with special care to the nature of the protecting groups on the serine acceptor, the pH and the temperature, has been performed.
The experimental work evidences that the synthesis yield increases upon protecting the serine molecule. α-*N*-Acetyl-galactosaminidase from *Acremonium sp.* turned out to allow shorter reaction times as compared to the enzymes of the same family reported in literature. Moreover, the yield values are competitive with those mentioned in previous works [4, 5].

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P044
BIOLOGICALLY ACTIVE GANGLIOSIDE GM3 MIMETIC POLYMERS

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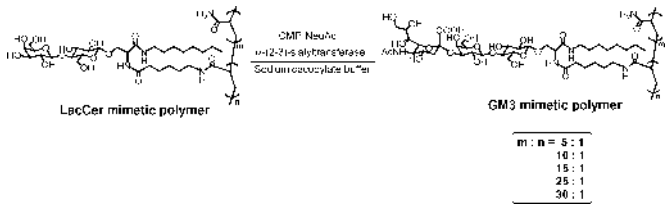
Ganglioside, a family of sialic acid-containing glycosphingolipids, have both various hydrophilic oligosaccharides chain and hydrophobic ceramide, and are known to be involved in cell growth, differentation, and signal transductions. Almost all of the ganglio-series gangliosides are synthesized from a common precursor, ganglioside GM3, which is a major one in the plasma membrane and is widely distributed in vertebrates. It is known that cell proliferation can be regulated by exogenous addition of GM3 at relatively high concentrations. We describe here the construction of GM3 mimetic polymers and their biological activities.
Five kinds of GM3 mimetic polymers having different sugar densities (1:5~1:30 sugar-acrylamide) were constructed from their water-soluble polymers (LacCer mimetic polymer) by using enzymatic method with sialyltransferase.



We found that the constructed GM3 mimetic polymers having sugar densities from 1:5 to 1:25 manifested notable growth inhibitory activity against various cancer cell lines, while the LacCer mimetic polymers did not. This result means that the sialic acid unit of the sugar chain is necessary to this bioactivity.

P045
CHEMICAL SYNTHESIS OF Tn ANTIGEN, A TUMOUR- ASSOCIATED CARBOHYDRATE ANTIGEN

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The stereoselective synthesis of glyco-aminoacids is quite challenging due to the high number and similarity of functional groups on the carbohydrate moiety. Various approaches have been reported for the chemical synthesis of tumour- associated antigens, including Tn (2-deoxy-2-acetamido-α-D-galactopyranosyl-*O*-serine). The most widespread strategy reports the use of glycals which are converted in the trichloroacetimidate after azidonitration, with a wide distribution of side-products, and a low steroselectivity (1:1 ratio of α and β anomers) [1-3].
This work reports the synthesis of Tn antigen using the chemistry of trichloroacetimidates obtained starting from 2-azido-2-deoxy-1,3,4,6-tetra-*O*-acetyl galactose [4]. The synthesis of trichloroacetimidate is initially followed by the

conjugation of the saccharide to the aminoacid (serine benzyl ester) and then by the removal of the protective groups. This strategy allows for a relatively high stereoselectivity (namely, a 3:1 ratio of α to β anomers is achieved), with a molar yield of 25%. An improved methodology aimed to the monitoring and purification of the α– and β– anomers of the product has been developed. It is based on high- performance liquid chromatography (amino- based stationary phases) with UV detection. This approach allows carrying out the one-step purification at the end of the reaction.

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P046

SYNTHESIS OF N-LINKED GLICANS AND GLYCOPEPTIDES TOWARD ANALYSIS OF GLYCOPROTEIN DEGRADATION PATHWAY

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Recent studies of glycoprotein quality control system revealed that Asn-linked high-mannose-type glycans play important roles in acquisition of correct folding and degradation of misfolded proteins. Asn-linked sugar chains are introduced cotranslationally in the endoplasmic reticulum (ER) as a tetradecasaccharide (Glc3Man9GlcNAc2). Glc3Man9GlcNAc2 is trimmed sequentially by glucosidase I and II to Glc2Man9GlcNAc2 and Glc1Man9GlcNAc2. Calnexin and its soluble homologue Calreticulin are molecular chaperons that recognize Glc1Man9GlcNAc2 and retain glycoproteins in the ER until they are successfully folded. In contrast, terminally misfolded glycoproteins destined for degradation are trimmed to Man8GlcNAc2, which is recognized by the α-mannosidase I-like protein and retrotranslocated into cytosol. Retrotranslated glycoproteins are ubiquitinated by SCF^{Fbs} complexes, where Fbs1 and Fbs2 are recently discovered F-box proteins that recognize sugar chains. Ubiquitinated glycoproteins are thought to be deglycosylated by peptide: N-glycanase and degradated by proteasome.

In order to quantitatively analyze various steps concerned with ER-associated glycoprotein degradation, we chemically synthesized a series of high-mannose-type glycans and glycopeptides. First, Manα1→6ManGlcNAc2, Manα1→3ManGlcNAc2, and Man3GlcNAc2 were synthesized and their affinities to Fbs1 were evaluated in comparison with previously synthesized Man9GlcNAc2 and Man3GlcNAc2. These analyses revealed that Man3GlcNAc2 had the strongest affinity and both the chitobiose and α1→6 linked Man residue are necessary for Fbs1 to recognize a sugar (Hagihara,S. *et. al.* J. Med. Chem. 2005, 3126-3129). Furthermore, Man3GlcNAc2 was conjugated to tripeptide (Asn-Gly-Thr), which constitutes N-glycosylation sequence. Asn(Man3GlcNAc2)-Gly-Thr had an affinity marginally weaker than that of Man3GlcNAc2, indicating that sugar-linked Asn residue and neighboring peptide are not strongly concerned with the recognition of Fbs1.

P047

ETHYL 2-THIO-β-D-FRUCTOFURANOSIDE 1-PHOSPHATE – A PRECURSOR OF THE POTENTIAL INHIBITORS OF GLYCOSYLTRANSFERASES

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Glycosyltransferases (GT's) belong to the enzymes that are involved in the biosynthesis of *N*- and *O*-linked complex oligosaccharides of glycoproteins. The reactions catalyzed by these enzymes form new glycosidic linkages. Modifications in the oligosaccharide chains accompany many physiological and pathological cell processes [1], and may lead to serious disorders in animals as well as in humans [2]. Therefore, inhibitors of these enzymes have a great therapeutical potential.

This contribution, aimed at developing and greater understanding of the transition state analog inhibitors of the GT's, introduces the synthesis of the simple precursor of this type – namely ethyl 2-thio-β-D-fructofuranoside 1-phosphate (1).

The starting point in the synthesis of intermediate 1 was ethyl 2-thio-β-D-fructofuranoside - which is readily available from D-fructose (in 4 steps – benzoylation, acetylation, thioglycosylation and deacylation) [3,4]. This thiosugar was blocked at position C-6 with terc-butyldimethylsilyl group [5] and following phosphoryla-tion with dibenzyl-chlorophosphate [6], desilylation with 1M tetrabutylammoniumfluoride in THF [5] and hydrogenation with Pd/C in methanol afforded the desired precursor 1 in satisfactory yield.

Acknowledgements: The work was supported by the Slovak Science and Technology Assistance Agency - APVT-51-004204

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P048

CHEMICAL SYNTHESIS AND IMMUNOLOGICAL ACTIVITY OF α-GALACTOSYLCERAMIDE DIMER.

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The synthetic glycolipid α-galactosylceramide (α-GalCer) enhances resistance to tumours and several intracellular infections. α-GalCer is presented by CD1d molecules (particularly on dendritic cells (DC)) to the CD1d-restricted Vα14-invariant NKT cells. This glycolipid acts as an adjuvant for T cell immunity in an NKT-dependent fashion by inducing maturation of DCs (up-regulation of CD40, CD80 and CD86 costimulatory molecules and MHC class II as well as producing large amounts of cytokines such as IFN-γ and IL-12). The α-GalCer/NKT system is attractive to study the role of different components of DC maturation *in vivo*.
To study this process we synthesized dimer of α-GalCer. This compound consists of two covalently linked molecules of α-GalCer through the ω-position of the fatty acids by "click chemistry" - the Huisgen 1,3-dipolar cycloaddition reaction between azide and acetylene. Preliminary results from *in vivo* experiments have revealed that the immunological activity of dimeric α-GalCer is significantly different from α-GalCer. Expression of the CD86 maturation marker by DCs *in vivo* in response to the dimer was 2- fold less than for α-GalCer, while B-cells showed the same level of CD86 for both compounds. The level of IL-12p70 in serum after α-GalCer dimer administration was also increased in comparison with α-GalCer. These data indicate that α-GalCer dimer may be useful as a new aduvant for the manipulation of immune responses *in vivo*.

P049
SYNTHESIS OF β-MANNOSIDES USING ENDO-β-MANNOSIDASE

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Endo-β-mannosidase [EC 3.2.1.152] is an endoglycosidase that hydrolyzes the Manβ1-4GlcNAc linkage of *N*-linked sugar chains. Recently, we purified this enzyme from *Lilium longiflorum* flowers and cloned a corresponding gene (1, 2). Here, we show that the lily endo-β-mannosidase has transglycosylation activity (3) and is useful for preparation of oligosaccharides containing β1-4-mannosides of which chemical preparations have been frequently reported to be difficult. In the presence of Manβ1-4GlcNAcβ1-4GlcNAc-peptides as a donor substrate and *p*NP β-GlcNAc as an acceptor substrate, the enzyme transferred mannose to the acceptor substrate by the β1-4 linkage regiospecifically and stereospecifically to give Manβ1-4GlcNAcβ1-*p*NP. Not only *p*NP β-GlcNAc but also *p*NP β-Glc and *p*NP β-Man worked as acceptor substrates. Besides mannose, oligomannoses were also transferred. In the presence of $(Man)_nMan\alpha1$-6Manβ1-4GlcNAcβ1-4GlcNAc-peptides (n=0-2) and pyridylamino GlcNAcβ1-4GlcNAc, the enzyme transferred $(Man)_nMan\alpha1$-6Man *en bloc* to the acceptor substrate to produce pyridylamino $(Man)_nMan\alpha1$-6Manβ1-4GlcNAcβ1-4GlcNAc.
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P050
MOLECULAR WEIGHT-TAGGED GLYCOPEPTIDE LIBRARY: EFFICIENT CONSTRUCTION AND APPLICATIONS

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Recently, glycoproteomics or glycomics has become a major academic and clinical research priority. Although oligosaccharides on glycoconjugates play vital roles in biological processes, the functions of glycoconjugates are little understood at the molecular level, mostly because of a lack of sensitive and high-throughput methods for analyzing the structure and interaction of oligosaccharides. To overcome these problems, technologies such as an observational multistage tandem mass spectral library [1] and arrays for carbohydrate and lectin have been developed. However, a large variety of structurally defined oligosaccharides, that is, an oligosaccharide library, is prerequisite for these technologies. Herein, we describe a method for the efficient construction of a glycopeptide library which can be easily converted to an oligosaccharide library by using human recombinant glycosyltransferases [2]. We developed a method for the construction of molecular weight-tagged libraries [3], in which the structure and molecular weight of the components were designed to correspond one-to-one by using glycosyltransferases. For achieving a diversity of oligosaccharides, by repeating incomplete enzyme reactions, it is possible to construct a library which has theoretically 2^n (n is the number of reaction steps) structures of oligosaccharides in a single tube. The structure of each component in the

library can be identified immediately by mass spectrometry alone because of each product has a different molecular weight. Furthermore, applications for the screening of ligand specificity for lectins by mass spectrometry are demonstrated.

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P051
ROLE OF GLUCOSE BONDING TO AMINO ACIDS IN MYELIN PROTEINS FOR AUTOANTIBODY RECOGNITION IN MULTIPLE SCLEROSIS

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The linkage unit to proteins of N-linked carbohydrates in eukaryotic glycoproteins is usually reported to be an N-acetylglucosamine coupled, by a β-bond, to the amido nitrogen of asparagine. Additional N-glycosyl linkage units have been, up to now proven to exist only in the cell surface glycoproteins of various bacteria. Among these units, glucose linked to asparagine was demonstrated to exist also in the mammalian protein laminin, an extracellular basement membrane component [1].
Moreover, an N-glucosyl linked unit made of glucose in α-anomeric form and Asn (α-GlcAsn) has been suggested to occur in eukaryotes [2], but this type of linkage has not been unequivocally proven to exist.
In this context, we demonstrated that the β-glucosylasparagine (β-GlcAsn) is fundamental for autoantibody recognition in Multiple Sclerosis (MS) in immunoenzymatic tests [3,4].
For that reason, we synthetized α-glucosylasparagine to be coupled to proteins and to obtain anti-(α-GlcAsn) antibodies to unequivocally state the anomeric selectivity, specific for MS.
Moreover glycopeptides bearing C-linked sugars are interesting mimics of naturally occurring O- or N-linked glycopeptides. They are attractive synthetic targets because of their increased stability to chemical and enzymatic cleavage. In fact, the C-glycopeptides are not subject to deglycosylation in vivo [5]. On this base, we synthetised *p*-glucosylphenylalanine with C-C linkage in β configuration, to show the role of this linkage type in autoantibody recognition in MS.

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P052
CHITINASE-CATALYZED SYNTHESIS OF CHITIN-CHITOSAN HYBRID POLYSACCHARIDE WITH WELL-DEFINED STRUCTURE

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Chitin and chitosan are well-known natural polysaccharides consisting of *β*(1→4)-linked *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN), respectively. They have excellent biodegradable and biocompatible characteristics in addition to their physiological activities like anti-inflammation and anti-bacterial activity. Therefore, they attract much attention as materials for medical and pharmaceutical sciences; numerous studies on physiological activities of chemically modified chitin and chitosan have been reported. However, structure-activity relationships of these polysaccharides are not discussed well due to their structural diversities derived from small portion of GlcN and GlcNAc moieties in natural chitin and chitosan, respectively, as well as unregulated reactions in the modification of these polysaccharide chains.
The present study demonstrated synthesis of chitin-chitosan hybrid polysaccharide, which has an alternating structure of *β*(1→4)-linked GlcN and GlcNAc. An *N*-acetyl chitobiose (GlcN*β*(1→4)GlcNAc) oxazoline derivative (**1**) was prepared as a transition state analogue substrate (TSAS) monomer for the catalysis of family 18 chitinases. The monomer **1** was effectively polymerized by the enzyme catalysis under weak alkaline conditions, providing a chitin-chitosan hybrid polysaccharide (**2**) in good yields. Molecular weight (Mn) value of **2** reached 1720, which corresponds to 10 saccharide units. Furthermore, chitinase-catalyzed copolymerization of **1** and *N,N′*-diacetylchitobiose oxazoline derivative (**3**) proceeded successfully; compound **3** has been reported as a TSAS monomer for the production of synthetic chitin. Composition of GlcNAc and GlcN units in the copolymers was controlled by varying the comonomer feed ratio from 1:1 to 1:0.

P053
CELL-TARGETING SYSTEMS: FROM THE GLYCOSYLATED CYCLODEXTRIN TO THE SPECIFIC RECOGNITION OF LECTINS WITH GLYCOLIGAND NANOAGGREGATES

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Specific recognition of cell-targeting systems is a major goal in the applications of supramolecular sciences to "drug delivery". One of the main challenge is to create self optimising cooperative structures having a controlled number of components, shape, and size able to target complexed drugs to the their biological site of action. Recently, it was demonstrated that specially designed amphiphilic β-cyclodextrins (CDs) are capable of forming micellar aggregates or bilayer vesicles as less immunogenic and more versatile drug encapsulators than a single CD molecule. Nanoaggregates of amphiphilic β-CDs with alkylthio chains of different length at the primary-hydroxyl side and glycosylthio-oligo-(etyleneglycol) units at the secondary-hydroxyl side were prepared. In particular nanoaggregates targeted by galactose show multivalent effects in their binding to lectins.[1] Here, by Elastic Light Scattering (ELS) and Cryo-TEM investigations, we describe in details structures and forms of glycoligand nanoaggregates in solution. AFM images display supported vesicles by casting glyco-CD on glass surface. Despite of the subtle differences in epitopes structures, by means of non invasive techniques (quasi elastic light scattering, QELS, and time resolved fluorescence) we focus on binding assessment of lectin from Pseudomonas Aeruginosa (PA-IL) by galactosylated CDs aggregates versus glucosylated CDs and the following extensive aggregation of the resulting CD/protein system. In conclusion we demonstrate the specific recognition of a protein can be tuned through the proper design of a smart vesicular glyco-carrier. All the results gave insights on the local protein microenvironments affected by glycoligand and furnished details on the diffusional dynamics of the interacting supramolecular species.

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P054
SYNTHESIS OF THE C-ANALOGUE OF SULFATIDE

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Introduction: The immune system is capable of recognizing lipid and glycolipid antigens presented by a family of antigen presenting molecules, the CD1 proteins. Sulfatide (**1**), a 3-O-sulfated β-D-galactosyl ceramide, is a promiscuous ligand that binds CD1 group I proteins and is presented by these molecules to specific T-cells.[1]
Materials and Methods: To better elucidate the antigen structural features influencing the recognition by CD1 and T-cells activation, the synthesis of the C-analogue of sulfatide was designed. The substitution of the anomeric oxygen by CH_2 might influence the formation of the CD1-glycolipid complex and the quality of T-cell response; moreover this replacement is expected to render the analogue resistant to galactosidases catabolism in vivo and consequently a different T-cell activity, due to a more persistent CD1-antigen complex, could be found.
Results: A new efficient synthesis of the C-analogue **2** of sulfatide is here reported. The key steps of the synthetic approach to **2** were a highly stereoselective Wittig rearrangement for the introduction of the β-C-galactosidic linkage and the Horner-Emmons olefination for the construction of the sphingoid skeleton carrying the desired E double bond.
Conclusions: A high stereoselective synthesis of a carbon-linked isoster of natural sulfatide was accomplished in good overall yield.
Acknowledgements: Authors thank MIUR-Itlay (COFIN2004-FIRB 2001) for financial support.
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P055
INTERACTION ANALYSIS BETWEEN GLYCOPOLYMERS CARRYING MULTIVALENT SIALYLOLIGOSACCHARIDES AND SIGLEC-2

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Siglec-2 is a B cell-specific transmembrane glycoprotein, which is known as a negative regulator of B cell receptor (BCR). The binding of mucins produced by epithelial cancer cells to Siglec-2 has been shown to partially inhibit intracellular signal transduction of BCR. In this study we have synthesized a series of glycopolymers possessing various lengths of sialylated *N*-acetyllactosamine- repeating oligosaccharides to construct artificial mucins that specifically bind Siglec-2 Interaction analysis between artificial mucins and human recombinant Siglec-2 has been carried out by ELISA.

N-acetyllactosamine-repeating oligosaccharide β-glycosides, Galβ1,4GlcNAcβ1,3Galβ1,4GlcNAcβ1,3Galβ1,4GlcNAcβ-*p*NP and Galβ1,4GlcNAcβ1,3Galβ1,4GlcNAcβ-*p*NP were synthesized by the alternative addition of β1,3-linked GlcNAc residue and β1,4-linked Gal residue to Galβ1,4GlcNAcβ-*p*NP, respectively, using human recombinant β1,3-*N*-acetylglucosaminyltransferase and bovine milk β1,4-galactosyltransferase. *p*-Nitrophenyl groups of $(Gal\beta1,4GlcNAc)_n\beta$-*p*NPs (n=1-3) were reduced by Pd/C. Resulting *p*-aminophenyl β-glycosides were coupled with γ-carboxyl groups of α-poly-L-glutamic acids with various lengths by condensation to form asialoglycopolymers possessing different molecular weights and degrees of substitution. The asialoglycopolymers were further converted into sialylated glycopolymers by commercially available rat recombinant α2,6-sialyltransferase or α2,3-sialyltransferase. Binding assay by ELISA has revealed that human recombinant Siglec-2 has specifically recognized glycopolymers carrying α2,6-linked Neu5Ac residues. On the other hand, hSiglec-2-R120A mutant lacking an essential Arg residue for binding to Neu5Ac residue did not recognize any sialylated glycopolymers. Furthermore, signal transduction through BCR has been partially inhibited by the treatment of sialylated glycopolymer carrying α2,6-linked Neu5Ac residues.

P056
SYNTHESIS OF THE COMMON TRISACCHARIDE FRAGMENT OF TWO GLYCOFORMS OF THE OUTER CORE REGION OF *PSEUDOMONAS AERUGINOSA* LIPOPOLYSACCHARIDE

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The core of the *Pseudomonas aeruginosa* lipopolysaccharide is recognized by receptors of the respiratory epithelial cells in cystic fibrosis patients. In order to ascertain which of two isomeric outer core region glycoforms is the ligand, we intend to synthesize the corresponding pentasaccharides representing these glycoforms. Here we describe preparation of their common branched trisaccharide methyl 2-(L-alanylamido)-2-deoxy-4-O-(α-D-glucopyranosyl)-3-O-(β-D-glucopyranosyl)-α-D-galactopyranoside and its 2-acetamido and 2-(N-acetyl-L-alanylamido) analogues. The latter was prepared to find out, whether the alanine amino group can be used for conjugates preparation without lost of binding activity. Two schemes of the oligosaccharide assembling were studied. The scheme including initial α-glucosylation at O-4 of the 2-azidogalactose block followed by β-glucosylation at O-3 failed, while the opposite order of glycosylation gave smoothly the protected trisaccharide. Removal of protecting group, reduction of the azido group and subsequent N-alanylation afforded the target trisaccharide.

This work was supported by DFG and the Russian Foundation for Basic Research, grant # 03-03-32-556.

P057
SUGAR–SCAN OF CSF114(GLC) FOR AUTOANTIBODY RECOGNITION IN MULTIPLE SCLEROSIS

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Protein glycosylation plays an important role in the biological activity of glycoproteins involved in antibody recognition, because they are exposed at the cellular membranes.In previous studies, we demonstrated that Asn(Glc) in the glycopeptide CSF114(Glc) [1-3] is the minimal epitope recognized by specific autoantibodies in sera of patients affected by Multiple Sclerosis (MS). No Abs could be identified using corresponding unglycosylated peptide sequence. Therefore, we could assess that the specific auto-antibody recognition is most likely driven by direct interactions of the antibodies with the Asn-linked sugar moiety. With the aim of developing CSF114(Glc) analogues, we synthesized this sequence containing different sugars. In fact, in the chemical synthesis of *N*-linked glycopeptides, it was demonstrated that the most versatile methodology is the stepwise approach, in which protected glycosylamines are coupled to Fmoc-protected Asp-residues to give Fmoc-Asn(Sug)-OR to be used in peptide synthesis.

Our aim is the optimization of the synthetic strategy adopted for a large scale preparation of the target molecules. In particular, the synthesis of natural sugars orthogonally protected for SPPS, and conjugation of the carbohydrate moieties to the side chain of Fmoc-Asp-OtBu are described. The efficiency of this approach will be demostrated introducing the new glycosylated amino acids in CSF114

analogues used to detect, by ELISA, antibody titre in MS patients sera.

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P058
SYNTHESIS OF CHONDROITIN SULFATE BY HYALURONIDASE-CATALYZED POLYMERIZATION

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Chondroitin sulfate (ChS) is one of the naturally occurring polysaccharides belonging to the glycosaminoglycan family, which exhibits critical bioactivities in vivo. ChS has a repeating structure of β(1→4)-linked β-D-glucuronyl-(1→3)-*N*-acetyl-D-galactosamine (GlcAβ(1→3)GalNAc; *N*-acetylchondrosine), whose hydroxyl groups are randomly sulfated. Particular glycosyltransferases are involved in the biosynthesis of skeletal ChS followed by sulfation by several kinds of sulfotransferases, leading to the structural diversity of ChS. However, detailed synthesis mechanism and physiological functions of ChS in vivo is not fully understood at a molecular level. The present study demonstrated hyaluronidase (HAase)-catalyzed synthesis of ChS with well-defined structure via nonbiosynthetic pathway. *N*-Acetylchondrosine oxazoline derivatives with sulfate groups at C4 (**1a**), C6 (**1b**) and both C4 and C6 (**1c**) in the GalNAc were prepared as transition-state analogue substrate monomers for HAase catalysis. Monomer **1a** was polymerized by the enzyme catalysis through ring-opening polyaddition under total control of regioselectivity and stereochemistry. The resulting polymer (**2a**) has a uniform structure bearing sulfate groups exclusively at C4 of all GalNAc units. Molecular weight value (M_n) of **2a** was controlled from 4,000 to 18,000 by varying pH conditions in the polymerization reaction, which agrees with that of naturally occurring ChS. In contrast, HAase only catalyzed the hydrolysis of **1b** and **1c** through the oxazoline ring-opening, affording the disaccharides without formation of the corresponding polymers. These results suggest that HAase recognizes sulfate groups for the catalysis activity during polymerization as well as hydrolysis.

P059
A UNIVERSAL APPROACH TO THE SYNTHESIS OF OLIGOSACCHARIDE—POLYHEDRAL BORON HYDRIDE CONJUGATES FOR LECTIN-MEDIATED TARGETING

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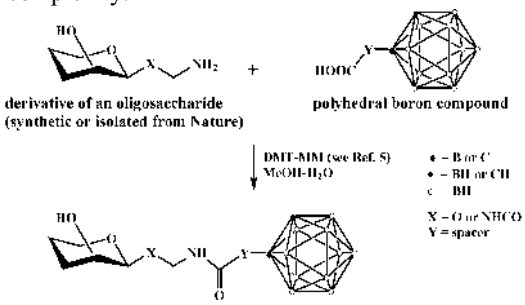
Polyhedral boron hydrides (PBHs) are promising agents for BNCT [1] and radionuclide diagnostics/therapy of cancer [2]. Their selectivity toward tumor tissues can be enhanced by using carbohydrate—PBH conjugates (CBCs), which comprise ligands of lectins over-expressed on tumor cells. All known syntheses of CBCs use *protected* mono- [3,4] or disaccharides [3].
We developed the first approach to the preparation of conjugates of *various* PBHs (*ortho*-carborane and *closo*-dodecaborate-anion) with *unprotected oligosaccharides* using lactose, a known ligand of lectins over-expressed on melanoma cells, as a representative example. The carboxy-derivatives of PBHs were coupled with *unprotected* amino-derivatives of *O*- or *N*-lactosides to give PBH-based neoglycoconjugates in good yield. This approach can be easily used for conjugation of oligosaccharides of almost any complexity.



This research was supported by RFBR (project No. 03-03-32622), the Program of the Presidium of the RAS "Directed Synthesis of Substances with Predetermined Properties and Development of Functional Materials Based on Them", the Program of the Division of Chemistry and Material Science of the RAS "Biomolecular and Medical Chemistry" and the Program for Support of Leading Scientific Schools of RF (grant NSh 1557.2003.3).

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P060
SYNTHESIS OF AN ANALOG OF THE REPEATING

UNIT OF CAPSULAR POLYSACCHARIDE OF *STREPTOCOCCUS PNEUMONIAE* TYPE 19F

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Introduction: Pneumococcal invasive infections are still a major problem both in developed and developing countries causing meningitis, otitis, septicemia and pneumonia. Among the different pathogens responsible for such infections, *Streptococcus pneumoniae* is one of the most virulent.
We are currently involved in a project devoted to the development of glycoconjugated vaccines through synthetic methods. We focused our attention on *S. p.* 19F whose CPS is composed of trisaccharide repeating units (→4-β-D-ManpNAc-(1→4)-α-D-Glcp-(1→2)-α-L-Rhap-1→) joined by phosphodiester bridges.
However, the presence of the phosphate on the anomeric position makes the polymer somewhat labile and the chemical synthesis of oligomer of the repeating unit difficult.
We then decided to substitute L-rhamnose with its carba-analog which, lacking the anomeric hydroxyl group allows to avoid the above problems.
Experimental and results: The synthesis of carba-L-rhamnose was described previously.(1) We performed the synthesis of the disaccharide β-D-ManpNAc-(1→4)-D-Glcp as follows: a properly protected glucoside acceptor was glycosylated with a glucosyl sulfoxide donor with a participating group at the position 2 to ensure the appropriate stereochemistry of the newly formed glycosidic linkage. Both donor and acceptor were obtained from a common intermediate. Inversion of the 2' position through the triflate-azide technique and further glycosylation afforded the desired trisaccharide analog.
Conclusion: In conclusion, we performed the synthesis of an analog of the repeating unit of *S. p.* 19F useful for the preparation of oligomer and conjugation to immunogenic proteins to evaluate its immunological properties.
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P061
SYNTHESIS AND BIOLOGICAL ACTIVITY OF GLUCOSE-DERIVED AKT/PI3K INHIBITORS

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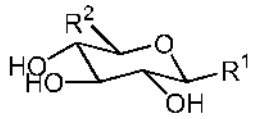
Phosphatidylinositol 3-kinase (PI3K) and Akt regulate many important cellular processes, controlling the balance of survival and apoptosis of mammalian cell. Akt is the major mediator of survival signals that protect cells from apoptosis, after activation in response to various stimuli, in a PI3K dependent manner. Recent studies showed the importance of Akt-PI3K pathway in many human diseases. In addition, Akt is a critical player in the development, growth and therapeutic resistance of cancer, thus emerging as therapeutic target for cancer treatment.
Although much of the drug-development efforts have been focused on ATP-binding-site inhibitors, development of small molecule analogues of natural phosphoinositides is now considered as potential alternative for pathway interruption and therapeutic applications.
In this context, compounds 1-4 have been synthesised starting from D-glucose, as potential inhibitors of the Akt-PI3K pathway as mimics of the natural phosphatidylinositol.
Carbohydrates are the most abundant chiral molecules naturally available. This characteristic, the low toxicity, low cost and easy with which they can be obtained in a pure state make these compounds as prime candidates for the synthesis of chiral scaffolds with pharmaceutical and medical applications.



1: R^1 – SOPh; R^2– CH_2OH
2: R^1 – $NHCOCH_3$; R^2– CH_2OH
3: R^1 – $NHPO(CH_2CH_3)_2$; R^2– CH_2OH
4: R^1 – $NHPO(CH_2CH_3)_2$; R^2– COOH

Compounds 1-4 were tested in inhibition assays on murine dendritic cells (DC). Compound 4 showed a 30 % inhibition of Akt/PI3K pathway, measured as reduction of interleukine 2 production, and Akt phosphorylation.

P062
EASY ACCESS TO GLYCOSYL PHOSPHOROTHIOATE WITH MICROWAVES TECHNIQUE

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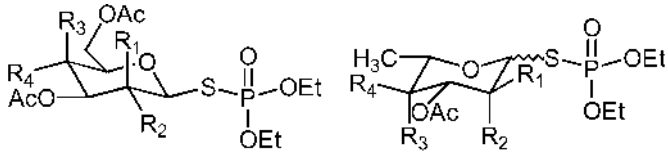
Glycosyl phosphorothioates, analogues of glycosyl phosphates, have shown attractive biological applications thanks to the significantly less reactivity towards anzymatic cleavages of the glycosidic bond. Recent studies have shown the pharmacological value of oligonucleotides analogues with

a phosphorothioate group as internucleotide linkage that confers stability to nucleases while preserving the specificity and efficiency of complexation. Furthermore phosphorothioate and phosphorodithioates possess most favorable biological and pharmacological properties as potential antisense and also as nucleic acids based clinical diagnostics.

Since the mid 1980s microwave activation as a non-conventional energy source has become a very popular and useful technology in organic chemistry, but only a limited number of microwave-assisted reactions have found application in carbohydrate chemistry.

In this context we describe a new, simple and efficient methodology for the synthesis of glycosyl phosphorothioates **1-4** by microwave dielectric heating, under solvent-free conditions. The reaction proceeds with good anomeric stereoselection.



1. R_1, R_3 = H; R_2, R_4 = OAc
2. R_2, R_3 = H; R_1, R_4 = OAc
3. R_1, R_4 = H; R_2, R_3 = OAc
4. R_1, R_3 = H; R_2, R_4 = OAc
5. R_2, R_4 = H; R_1, R_3 = OAc

The phosphorothioate formation proceeds through a one-pot reaction of the per-*O*-acetylated-α-glycosyl bromide with diethyl phosphite in the presence of an equimolar amount of ammonium acetate and sulphur, adsorbed on alumina. Glucosyl, galactosyl, and fucosyl phosphorothioate (compounds **1, 3** and **5,** respectively) were obtained in excellent yield (80-90%), while mannosyl and rhamnosyl phosphorothioates (**2** and **4**) were isolated only in 30 % yield, in agreement with related compounds previously reported.

P063
SYNTHESIS OF GLUCOSYLATED PEPTIDES: ROLE OF THE AMINO-ACID SEQUENCE IN AUTO-ANTIBODY RECOGNITION IN MULTIPLE SCLEROSIS PATIENTS' SERA

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The glycopeptide CSF114(Glc) [1], characterized by a β-turn motif [2] typical of a glycosylation site, was synthesized after a structure-based design aimed to confirm that the role of the amino-acid sequence is not only relevant, but essential, to a correct presentation of the sugar moiety to be recognized by the autoantibodies. The designed glycopeptide CSF114(Glc) was able to detect the best autoantibody titre in Multiple Sclerosis patients' sera in solid phase-enzyme linked immunosorbent assay (SP-ELISA) [3, 4].

In previous studies [5], we found that Asn(Glc) was fundamental to detect autoantibodies MS patients' sera. To optimize the length of the synthetic mimetic antigen to be used in SP-ELISA, we synthesized several glycopeptides, all containing Asn(Glc), shortened at both termini to find out the minimal glycopeptide sequence.

Increased antibody titres in SP-ELISA were detected with the longer glycopeptide sequences, while all the shortened glycopeptides inhibit anti-CSF114(Glc) autoantibodies in competitive ELISA. These results demonstrated that the length of the synthetic antigen is fundamental, particularly for the coating of the antigen to the polystyrene of the ELISA plate. The sequence of the amino acids involved in the β-hairpin conformation is crucial for the autoantibody recognition event. Our attention was then focused to minimal consensus sequences for asparagine-glycosylation in N-glycoproteins. We synthesized a library of possible sequences Asn-Xaa-Ser/Thr, which were tested in competitive ELISA.

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P064
CHEMICAL SYNTHESIS OF SIALIC ACID POLYPRENYL PHOSPHATE, A PROBABLE BIOSYNTHETIC INTERMEDIATE OF BACTERIAL POLYSIALIC ACID

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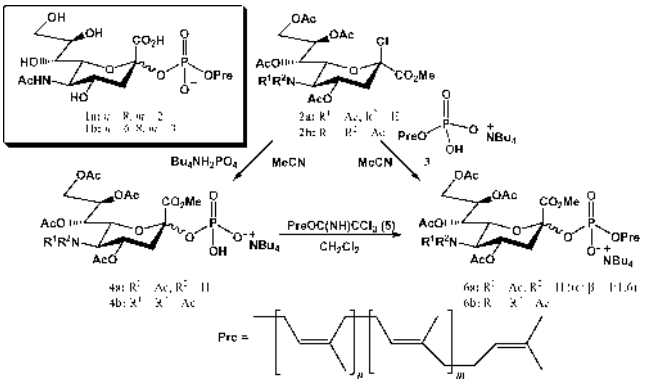
Sialic acids play important roles in many biological events. Polymeric forms of sialic acids are known (PSA) [1]. PSA are composed of *N*-acetylneuraminic acid (Neu5Ac) residues joined internally by α-(2-8)-, α-(2-9)- or alternating α-(2-8)-/α-(2-9) linkages. Although biosynthesis of bacterial PSA has been thoroughly studied, some details are still ambiguous. Neu5Ac undecaprenyl phosphate (**1a**) was postulated to be the key intermediate of the biosynthesis [1]. This compound has never been isolated and characterized. The exact stereochemistry of **1a** is unknown.

To elucidate this point, we attempted a synthesis of α- and β-isomers of Neu5Ac moraprenyl phosphate (**1b**) using the known Neu5Ac chloride **2a** and the novel $Neu5Ac_2$ chloride **2b** [3].



We found that acetylated Neu5Ac moraprenyl phosphate (**6a**) could be prepared directly from sialic acid chloride **2a** and tetrabutylammonium moraprenyl phosphate (**3**) in MeCN (α:β = 1:1.6). The α-isomer of Neu5Ac moraprenyl phosphate (**α-6a**) may also be obtained from the known acetylated Neu5Ac α-phosphate (**α-4a**) [2]. Chloride **2b** was also involved in similar reactions.

This research was financially supported by Russian Foundation for Basic Research (projects 04-03-32854 and 05-03-32579) and by the State Program for the Support of Leading Scientific Schools of the RF (Grant NSh 1557.2003.3).

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P065
TOWARDS BISUBSTRATE-TYPE INHIBITORS OF TRANS-SIALIDASES

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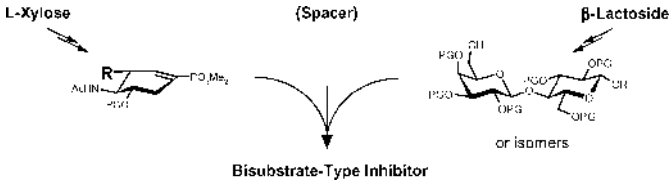
Trypanosomal trans-sialidases transfer sialic acid from the host cell glycocalix to the parasite's own cell surface glycoconjugates, thus conferring altered physicochemical properties on the respective trypanosomal parasite which are essential for survival in the host.[1]
In the case of the trans-sialidase from *T. cruzi*, which is pathogenic to man, a double displacement mechanism of the sialyltransfer has been proposed and the crystal structure of a ternary complex between the enzyme, the sialylmimetic DANA which is a weak inhibitor, and the acceptor substrate lactose has been solved.[2-4]
The finding that the enzyme requires acceptor-substrate binding to shape the active site for full sialidase activity prompted us to develop a synthesis of bisubstrate-type inhibitors which combine the features of classical influenza sialidase inhibition and acceptor substrate binding.
We will report on the synthesis of *xylo*-configured cyclohexenephosphonates[5,6] as sialylmimetics linked to, as a monoester, suitable acceptor substrates (fig. 1). Optionally, a spacer moiety can be inserted.



We will report on the chemical synthesis of the inhibitors and, if available, new data on the inhibition of *T. cruzi* trans-sialidase will be included.

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P066
SYNTHESIS OF BETAGLYCAN-TYPE TETRAOSYL OLIGOPEPTIDES AS THE PRIMERS REGULATING ENZYMATIC ELONGATION TOWARD SPECIFIC GLYCOSAMINOGLYCANS

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Glycosaminoglycans (GAGs) are classified into two categories based on the types of hexosamine residue in the repeating region; heparin and chondroitin, which have α-GlcNAc and β-GalNAc, respectively. The detailed mechanisms of the first addition of the hexosamine to the tetrasaccharide (GlcAGalGalXyl) at the linkage region have been obscure. However, the fact that the heparin-type GAGs often link to hydrophobic and acidic region of the core-peptide[1)] might deeply relate to the sorting mechanisms of GAG. We have synthesized some tetraosyl oligopeptides which are partial sequence of betaglycan to investigate the environmental effects derived from the core-peptide for the first hexosamine transfer to GlcA residue. Based on a retrosynthetic analysis we divided the target small proteoglycans into three parts: GlcAGalGalXyl, SerGly and the other peptide sequences. Tetraosyl imidate was glycosylated with the free hydroxyl group of the SerGly.

Then, liberated C-terminal of the tetraosyl dipeptide and the N-terminal of the appropriate oligopeptides in liquid phase or on solid support were coupled successfully. Following deprotection and purification procedures finally afforded the desired compounds. Currently these glycosyl oligopeptides are under investigation as the acceptors for enzymatic transfer of hexosamine residues.

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P067
SYNTHESIS OF 2-ACETYLAMINO-DEOXYGALACTONOJIRIMYCIN DERIVATIVES

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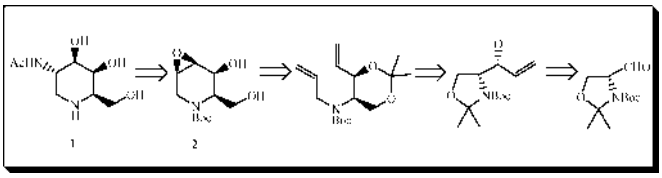
Azasugars are inhibitors of carbohydrate-processing enzymes and could be of value for treatment of diseases such as lysosomal storage disorders [1].
We are interested in derivatives of 1-Deoxygalactonojirimycin as potential inhibitors of N-Acetylgalactosamine-transferring enzymes. Such compounds are of value for the treatment of GM2-gangliosidoses, either as glycosyltransferase-inhibitors within the concept of substrate reduction therapy, or as glycosidase inhibitors as Chemical Chaperones for enzyme enhancement therapy. For this purpose we required the title compound **1** in the D-galacto configuration [2].



Scheme 1: Retrosynthesis of 2-Acetylamino-deoxygalactonojirimycin **1**

Since larger amounts of the title compound **1** were required for structure activity relationships, we developed an efficient approach for the synthesis of **1** from D-Garner aldehyde according to scheme 1. The epoxide **2** is available from D-serine in 8 steps [3]. The regioselective ring opening of the epoxide **2** afforded the title compound and allows preparation of N-alkylated analogs.

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[2] T. Kolter and M. Wendeler, *ChemBioChem*, **2003**, *4*, 260-264
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P068
ENHANCED SIALYLATING ACTIVITY OF O-CHLOROACETYL 2-THIOETHYL SIALOSIDES. SYNTHESIS OF A SPACERED GD3 TETRASACCHARIDE FOR GLYCOCONJUGATE DESIGN

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The disaccharide sequence Neu5Acα(2-8)Neu5Ac is a constituent of a number of gangliosides such as GD_3, GT_{1a}, GQ_{1b}, and others, and plays a key role in their biological activities. Chemical preparation of the Neu5Acα(2-8)Neu5Ac dimer is strongly complicated by the low reactivity of the 8-OH group of neuraminic acid and remains so far one of the main problems of the oligosaccharide synthesis. It has been shown that 2-thioethyl sialosides with electron-withdrawing O-chloroacetyl protecting groups are considerably more reactive and provide higher yields and α-stereoselectivity in sialylation than conventional O-acetylated sialyl donors. Thioethyl sialoside that combines electron-withdrawing *O*-chloroacetyl and *N*-trifluoroacetyl protecting groups displayed the best reactivity and enabled the most efficient so far synthesis of the Neu5Acα(2-8)Neu5Ac dimer. Fully and selectively O-chloroacetylated thioethyl sialosides [methyl(ethyl 4,7,8,9-tetra-O-chloroacetyl-3,5-dideoxy-2-thio-5-trifluoroacetamido-D-*glycero*-α-D-*galacto*-non-2-ulopyranoside)onate and methyl(ethyl 4-O-benzoyl-9-O-benzyl-7,8-di-O-chloroacetyl-3,5-dideoxy-2-thio-5-trifluoroacetamido-D-*glycero*-α-D-*galacto*-non-2-ulopyranoside)onate] were applied to the synthesis of the carbohydrate portion of the GD3 ganglioside. Successive and block approaches were explored. In the former, two neuraminic acid residues were introduced into the oligosaccharide chain stepwise. In the latter, the initially prepared disaccharide Neu5Acα(2-8)Neu5Ac was converted into a dimeric sialyl donor and then coupled with a lactoside acceptor. The first chemical synthesis of the neuraminic acid α(2-8)-trimer has been carried out with the use of the above chloroacetylated sialyl donors.

This work was supported by the Russian Foundation for Basic Research, grant # 03-03-32567.

P069
SYNTHESIS OF OLIGO-β-(1-6)-N-ACETYLGLUCOSAMINES, FRAGMENTS OF THE POLYSACCHARIDE INTERCELLULAR ADHESIN OF *S. AUREUS* AND *S. EPIDERMIDIS*

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It has been shown recently that antibodies against partially de-N-acetylated β(1-6)-linked poly-N-acetyl glucosamine (PNAG) surface polysaccharide antigen mediated effectively

killing of a variety of strains of *S. aureus* and *S. epidermidis*. However, the exact chemical nature of the protective epitopes is not known. To define the exact chemical structure of the most important fragments giving rise to protective immunity, chemical synthesis of oligoglucosamines with glucosamine units bearing N-acetylated and free amino groups in defined places was necessary. As a first step towards this goal, preparation of oligosaccharides either with all N-acetylated or all N-unprotected glucosamine units was studied. First, oligomerization of mono- or oligosaccharide glucosamine derivatives that contained both glycosyl donor and glycosyl acceptor sites was explored. However, this approach afforded mainly cyclic products of intramolecular glycosylation and only low yield of linear β(1-6)-glucosamines. Another approach consisted in step-by-step elongation of the oligosaccharide chain with the use of di- or tetrasaccharide glycosyl donors. It provided a series of protected higher β(1-6)-oligoglucosamines. Subsequent removal of protecting group with or without N-acetylation resulted in the formation of fully N-acetylated or N-unprotected oligoglucosamines, respectively. Protecting groups pattern allowed preparation of oligosaccharides containing thiol functionality in a spacer group for further conjugation with a protein carrier. A set of orthogonal N-protecting groups for preparation of β(1-6)-glucosamines having N-acetyl groups in defined glucosamine residues and thiol in a spacer-arm has been elaborated.

The work was supported by CRDF and the Russian Foundation for Basic Research, grant # 05-03-08107.

P070
GLYCOSYLATED PROTEINS OF THE GASTRIC PATHOGEN *HELICOBACTER PYLORI*

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The aim of this study was to detect the glycosylated proteins of *Helicobacter pylori* separated by one (SDS-PAGE) and two dimensional gel electrophoresis (2-DE) techniques. The Gram negative bacterium *Helicobacter pylori* is a human pathogen which infects the gastric mucosa and causes an inflammatory process leading to gastritis, ulceration and cancer. Protein analysis of the *Helicobacter pylori* strains (PA4, PR20, P12) was performed using denaturing 15% or 8% SDS-PAGE according to the method of Laemmli. *Helicobacter pylori* cell proteins were also separated by 2-DE into hundreds of spots with small and large gel techniques. Separated proteins of *Helicobacter pylori* strains by SDS-PAGE and 2-DE were transferred to PVDF or nitrocellulose membranes by semi-dry blotting technique. Detection of glycosylated proteins of the protein bands or spots on blotted membranes were determined by overlay reactions with Digoxigenin (DIG) Glycan Detection kit (Roche Diagnostics, Germany). The Roche DIG-Glycan Differentiation Kit (lectin protein detection with GNA, SNA, MAA, PNA and DSA) was also utilized to further characterize the glycosidic modifications.

Analysis of protein spots on membranes with DIG Glycan Detection kit after SDS-PAGE analysis gave the general pattern of glycosylated proteins of *Helicobacter pylori*; interestingly PA4, PR20 and P12 strains of *Helicobacter pylori* gave the different patterns of protein glycosylation on 8% polyacrilamide gels. Moreover, a glycosylated protein band (~54kDa) was also detected dominantly on the outer membrane part of *Helicobacter pylori*. Structural characterisation of the carbohydrate chains of glycoproteins bound to membrane, which have been separated by 2-DE and transferred, suggest that *Helicobacter pylori* proteins contain mainly O-glycosidically linked carbohydrate chains as determined by PNA lectin staining. Two dimansional electrophoretic analysis of *Helicobacter pylori* proteins showed about twelve clear spots indicating the O-glycosidically linked carbohydrate chains by PNA lectin staining of the blotted membrane. Moreover, MAA, GNA and DSA lectin stainings of the blotted membranes after two dimansional gel electrophoresis gave weaker spots belong to PA4 strain of *Helicobacter pylori* respectively. These results suggest the precence of some potential glycosylated proteins in *Helicobacter pylori*. Future studies should concentrate to characterise and describe in more detail for the possible roles of these potentially glycosylated proteins of *Helicobacter pylori* in its pathogenicity.

P071
SYNTHESIS OF CONFORMATIONALLY RESTRICTED CERAMIDE ANALOGS AND THEIR EFFECT ON GLYCOLIPID METABOLISM

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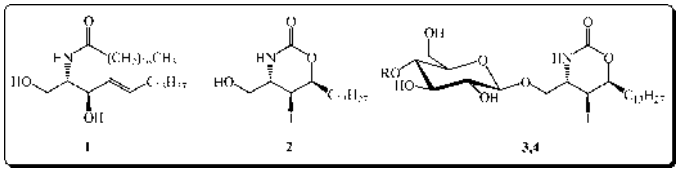
Ceramide (**1**) is a structural component of membrane glycosphingolipids.[1] Exogenous application of ceramide and ceramide analogues to cultured cells can lead to a variety of effects, including metabolic transformation and apoptotic responses.[2]

The restriction of conformational flexible biomolecules is a successful strategy in drug development. We report on the synthesis and selected properties of heterocyclic ceramide analogues like **2** shown in figure 1.



Structures of ceramide **1**, a synthetic ceramide analogue **2**, synthetic neoglycolipids **3** (R = H) and **4** (R = Gal β1,4)

Addition of 2 to cultured neuronal cells led to a decrease in levels of *N*-Acetyl-galactosaminyltransferase (GalNAc-T) products. This enzyme converts ganglioside GM3 (NeuAcα2,3Galβ1,4Glcβ1Cer) into ganglioside GM2 (GalNAcβ1,4(NeuAcα2,3)Galβ1,4Glcβ1Cer). We investigated the effect of the lipids 2-4 on GalNAcT in a micellar enzyme assay. The results of this study are reported.

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P072
AUTOMATED GLYCOSYNTHESIZER "Golgi™"

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Introduction
As biological importances of glycoconjugates become clear, the efficient synthetic method of glycoconjugates come to be demanded. In our previous studies, we have proposed the enzymatic glycoconjugate synthesis on a water-soluble primer polymer, and we have tried to carry out it automatically on a glycosynthesizer "Golgi™". These primer polymers were designed to be able to release carbohydrates which synthesized on it, and chemically prepared. On the other hand, immobilized glycosyltransferases which were prepared with very simply and quickly techniques, or un-immobilized ones used in this study.
Experimental
Some glycosyltransferases were expressed in *E. coli* as fusion proteins with maltose-binding-protein (MBP), respectively. Through the purification processes of glycosyltransferases, these recombinant enzymes can be immobilized on the surface of magnetic beads carrying affinity ligand for MBP. Primer-polymers having photo-labile linkage as a releasing point of glycopeptide were chemically synthesized (Fumoto *et al*; *Angew Chem Int Ed Engl*. 2005 Apr 22; 44(17):2534-7.). Primers were consisted of polyacrylamide and biomimetic glycopeptides. Primers, sugar-nucleotides and enzymes were set in sample vessels of "Golgi™", and enzymatic glycosylation reactions were performed. "Golgi™" could control reaction time, temperature and scale, and purify products by ultra-filtration automatically.

In this research, we also designed and produced a new "Golgi™". The latest model is suitable to use magnetic beads and heat-sensitive reagents.

P073
CONFORMATIONAL STUDIES OF A FIFTEEN AMINO ACID RESIDUE PEPTIDE BINDING TO THE GM1 OLIGOSACCHARIDE

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A peptide with a sequence of VWRLLAPPFSNRLLP displayed a clear structural alteration between in the presence of and in the absence of GM1 oligosaccharide. The binding affinities between the peptide and oligosaccharides were measured by the quartz crystal microbalance (QCM) and the coldspray ionization mass spectrometry (CSI-MS) methods, and the three-dimensional structures of the peptide with/without the GM1 oligosaccharides were analyzed using two-dimensional nuclear magnetic resonance (NMR) experiments with distance-restrained simulated annealing calculations.
The NMR experiments indicated that the peptide has two conformers derived from the exchanges between *cis* and *trans* forms in the portion of Pro-Pro, and has a bended conformation without the regular secondary structures. Further NMR studies, on the other hand, with the complex of the peptide and GM1 oligosaccharides elucidated the *trans* form of the peptide bound to GM1, and the peptide showed a highly stable structure stabilized by the hydrophobic interactions including β- and helical turns. These results were also supported by computational calculation results.
Based on these structural investigations, the tryptophan, a core residue of the hydrophobic cluster, might be an essential residue for the recognition of the GM1 oligosaccharides. The dynamic transition of the ligand peptide might play an important role in the function of GM1 as a multiple receptor such as the traditional pathway of the infection of cholera toxin.

Reference. FEBS Lett., ***456****, 253-256 (1999).*

P074
EFFICENT ENZYMATIC SYNTHESIS OF THE DERIVATIVES OF NACETYLLACTOSANIDE AND SIALYL N-ACETYLLACTOSAMINIDES BY β–GALACTOSIDASE AND SIALYLTRANSFERASE

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The oligosaccharide components in glycoproteins, glycolipids, and polysaccharides play crucial roles in many important biological processes, including cell-cell recognition and interaction, growth regulation, differentiation, and adhension. Activities of these oligosaccharides are generally regulated by the sequential actions of glycosidases and glycosyltransferases in the Golgi apparatus. Neuraminidase is one of glycosidases that cleaves *N*-acetylneuraminic acid from the terminal position of glycoproteins. For influenza virus, the neuraminidase on the surface of virus is an attractive target for antiviral intervention and the focus for rational drug design as its active site remains unchanged during genetic drifts and shifts. Sialyltransferase catalyzes the transfer of sialic acid residue from cytidine-5'-mono-phosphate-*N*-acetylneuraminic acid to the nonreducing end of the oligosaccharide. The increasing or decreasing its quantity in the cell may be related to pathogens, proliferation, and differentiation. Therefore, facile and efficient assays for glycosidase and glycosyltransferase are of growing importance. Furthermore, the need for practical synthetic procedures of oligosaccharides and their analogs in large quantities has become a major subject as the understanding of these biological functions increases. Here, we report the enzymatic synthesis of 4-methylumbelliferyl or *p*-nitrophenyl derivatives of *N*-acetyllactosaminide and sialyl *N*-acetyllactosaminide by combination of b-galactosidase and sialyltransferases. The synthesis of 4-methylumbelliferyl *N*-acetyllactosaminide (MU-LacNAc, **1**) and *p*-nitrophenyl *N*-acetyllactosaminide (*p*NP-LacNAc, **2**) were conveniently done by the transglycosylation of β-galactosidase from *Bacillus circulans*. Before use, the crude β-galactosidase was partially purified by hydrophobic interaction chromatography on a column of high substitued Phenyl Sepharose 6 Fast Flow to remove the contained β-acetylhexosamindase. And the sialylation of **1** and **2** were carried out with recombinant rat α2,3-(*N*)-sialyltransferase and rat liver α2,6-(*N*)-sialyltransferase to afford α2,3-linked (**3** and **4**) and α2,6-linked (**5** and **6**) sialyl *N*-acetyllactosaminides, respectively. Compounds **1**, **3**, and **5** will be quite useful to investigate the activities of neuraminidase, sialyltransferase, and fucosyltransferase, while other compounds **2**, **4**, and **6** will be useful for the study of specific carbohydrate-carbohydrate and carbohydrate-protein interactions with quartz crystal microbalance or surface plasmon resonance methods.

P075
HIGH AFFINITY AND SPECIFIC CARBOHYDRATE-ENCAPSULATED GOLD NAPOPARTICLES FOR TARGET PROTEIN IDENTIFICATION AND BINDING EPITOPE MAPPING

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The interactions of cell-surface glycoproteins and glycolipids play important roles in cell-cell communication, proliferation and differentiation. Thus, study of carbohydrate-related interactions might provide new insights into their biological roles and reveal new possibilities for drug development and detection methods. Disclosure of the carbohydrate-recognition sites by X-ray crystallography and NMR spectroscopy has been a challenge due to the difficulty of cocrystallization of targeting proteins and carbohydrates. We report here a new approach by using carbohydrate-encapsulated gold nanoparticles(c-AuNP) as an affinity probe for the efficient separation and enrichment of target proteins (PA-I and SLT IB) and then protein identification and epitope mapping by MALDI-TOF MS. The affinities between c-AuNP and target proteins were investigated by using Surface Plasmon Resonance (SPR) study. Due to the multivalent interaction between PA-I and Gal-AuNP, highly affinity and specific separation was achieved, and current detection limit is 0.78 nM PA-I in 100 uL mix protein pool, which is equivalent to 78 fmole of PA-I. The Gal-AuNP captured PA-IL was subjected to direct in situ digestion by chymotrypsin and carbohydrate-binding site of PA-IL was located precisely. The mass spectrum revealed that R83-Y105, A1-Y36, and R83-S121 three domains of PA-I were involved in the specific recognition with Gal-AuNP. Also, P^k-AuNP captured SLT-IB results indicated that E48-K73 of SLT-IB was the specific recognition domain for P^k-AuNP. The K_is of 4, 13 and 20 nm P^k-AuNPs with PA-IL are 10^{-9}, 10^{-10} and 10^{-11} M, respectively, while those with SLT-IB are 10^{-11}, 10^{-12} and 10^{-13} M, respectively. In summary, we have demonstrated the feasibility of carbohydrate-functionalized nanoparticle for enrichment and isolation of target protein, and mapping the binding-epitope containing sequence. The multivalent interaction between target protein and P^k-AuNP is affected by particle size, linker length and binding model.

P076
GLYCONAPOPARTICLES: NEW NANOMATERIALS FOR GLICOSCIENCE STUDIES

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Effectively designing model systems to study biological interactions and mediate in biological processes requires careful consideration of the special characteristics of the system to be mimicked (1). Searching for appropriate multivalent systems to compensate for the low affinity of the carbohydrates' interactions, we have prepared gold, magnetic and fluorescent nanoparticles containing carbohydrates on their surface. These glyconanoparticles have been obtained by a simple strategy and have a globular and polyvalent carbohydrate display. Different metal or semiconductor cores have been used for obtaining glyconanoparticles with different physical properties:

a) Gold glyconanoparticles prepared with Le^x-antigen and lactose have been used to study and evaluate the selective self-recognition of the Le^x antigen via carbohydrate-carbohydrate interactions, and to clarify the involvement of the interaction GM_3-lactose glycosphingolipids in metastasis processes by in vitro studies (2).

b) Gold-iron glyconanoparticles (MGNPs) prepared with glucose, maltose and lactose, have the same properties as gold glyconanoparticles, but a small iron amount has been introduced in the metal core. These molecules are superparamagnetic (3) and the preliminary results of MGNPs capable of crossing the Blood Brain Barrier, their accumulation in brain tumours and their visualization by MRI, have demonstrated their potential application in Biomedicine.

c) Luminescent CdS nanocrystals prepared with maltose can be used as bioactive fluorescent probes in diagnostics applications (4). Compared with conventional fluorophores, the nanocrystals have a narrow, tunable and symmetric emission spectrum.

All these glyconanoparticles may be considered as models to mimic glycosphingolipid clustering on the plasma membrane, and they are a very useful polyvalent carbohydrate display for different biological studies.

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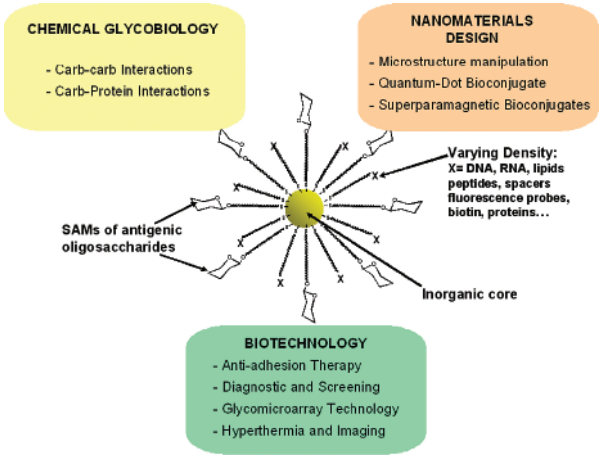

Figure 1. The glyconanoparticle concept and its potential applications.

P077
EFFECTIVE PROTEIN AND SOLID SURFACE MODIFICATION VIA 1, 3-DIPOLAR CYCLOADDITION

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Effective and site-specific modification is always an important issue for bio-conjugation research and solid surface study. However, many biomolecules need to be dissolved in aqueous solution for incorporation with proteins or solid supports. The Cu (I)-catalyzed 1,3-dipolar cycloaddition provides a powerful method for bioconjugation in aqueous buffer through formation of cyclic triazole. This post presents the use of intein protein overexpression system to prepare the C-terminal alkyne-modified protein and then ligates with other azide containing molecules, including glucosamine, glycopeptide, enhanced green fluorescent protein (eGFP), FITC and biotin. Incubation of $CuSO_4$, Tris(2-carboxyethyl) phosphine (TCEP), and tris(triazolyl) amine ligands and alkyne modified MBP in PBS (0.1M, PH 8) for 6 hours at 25□ gave the desired conjugates which were identified by ESI mass spectrometry, in Figure 1.. Based on the same type of reaction, effective modification on solid support was demonstrated by cycloaddition of alkyne containing biomolecules with azide modified surface such as glass-slide and magnetic nanoparticle via 1,3-dipolar cycloaddition. In this study, we exploited a practical and convenient method to modify the protein and solid support surface in aqueous solution via Cu (I)-catalyzed 1, 3-dipolar cycloaddition.

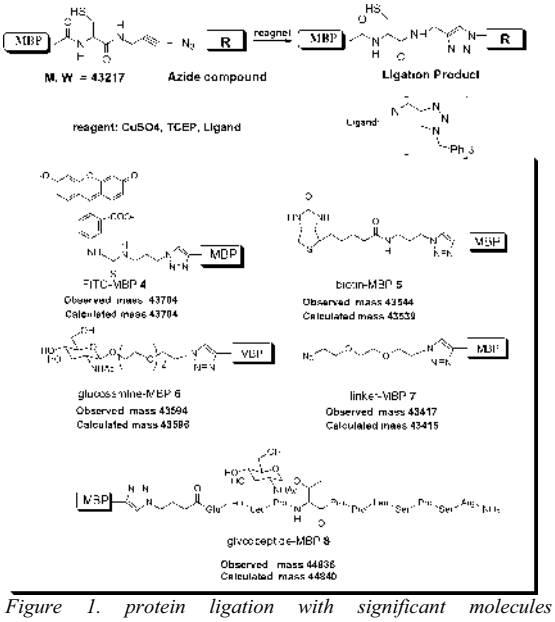

Figure 1. protein ligation with significant molecules via 1,3-dipolarcycloaddition

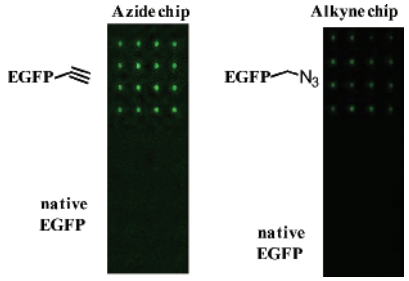

Figure 2. 1,3-dipolar cycloaddition on glass slide

P078
OLIGOSACCHARIDE LIGANDS FOR THE β-GLUCAN RECEPTOR, DECTIN-1, ASSIGNED BY THE NEOGLYCOLIPID-BASED MICROARRAY TECHNOLOGY

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Dectin-1 is a receptor of the immune system on leukocytes with a lectin-like domain of the C-type (Ca^{2+}-dependent) family[1]. Dectin-1 lacks residues involved in calcium ligation that mediates carbohydrate binding by classical C-type lectins, nevertheless, it binds zymosan, a particulate β-glucan-rich extract of *Saccharomyces cerevisiae*; binding is inhibited by polysaccharides rich in β1-3- or both β1-3- and β1-6-linked glucose. Dectin-1 is the major receptor on leukocytes for fungal β-glucans, and is involved in phagocytosis and triggering of inflammatory mediator production in innate immunity to these pathogens. In this study we have used a recombinant soluble form of Dectin-1 to investigate the oligosaccharide ligands on β1-3 or β1-6 glucose oligomers by generating oligosaccharide probes, neoglycolipids[2], from three polysaccharides: a neutral soluble glucan (NSG) isolated from *S. cerivisiae,* and two bacterial glucans, curdlan from *Alcaligenes faecalis* and pustulan from *Umbilicaria papullosa*. We have furthermore evaluated Dectin-1 binding to a microarray of over 100 mammalian-type oligosaccharide probes (neoglycolipids and glycolipids), and observed binding only to the 1-3-linked glucose oligomers. We shall describe the minimum chain length required for detectable binding, and also results of experiments which show that Dectin-1 ligands, in clustered form (displayed on liposomes) compete with zymosan binding and triggering of TNF-α secretion by a macrophage cell line, which expresses high levels of Dectin-1.

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Biotechnology in glycoconjugates research

P079
MULTIFUNCTIONAL CARBOHYDRATE PHOTOAFFINITY PROBE CARRYING A NOVEL CLEAVABLE BIOTIN

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Biotin-bearing multi-functional photoaffinity probes are powerful tools for the identification of receptor proteins and their binding sites.[1] A photoreactive *N*-acetylglucosamine carrying biotinylated diazirine photophore provided the first information regarding acceptor site peptides of β1,4-galactosyltransferase. Here we wish to report an efficient approach for fishing-out the target protein from a protein mixture.

The method utilizes diazirinyl carbohydrate ligands connecting a biotin tag through a novel cleavable acylsufonamide linker. Acylsulfonamide is known to be a useful linker for solid-phase peptide synthesis as well as combinatorial synthesis whereas the application of acylsulfonamide chemistry was optimized only in non-aqueous conditions and biological application of the acylsulfonamide group has not been exploited. After

photoaffinity biotinylation of *Ricinus communis* agglutinin, using a diazirinyl Galβ-1,4-GluNAc photoprobe carrying the cleavable biotin, the cleavage step was examined at various conditions. The acylsulfonamide linkage was chemically very stable to allow various harsh handling of proteins without the loss of biotin tag. Upon *N*-alkylation, the linkage is efficiently cleaved in 0.1 M borate buffer at pH 9.0 with 0.02 M NH_3. The method enabled the specific isolation of galactose binding B subunit from a photolabeled sample mixture by the application of trap-and-cleave sequence combined with avidin matrix. Non-specifically adsorbed proteins on the matrix were not released.

This approach may extend the potential of photoaffinity labeling to become a more sensitive means for rapid elucidation of protein structures and binding sites.

[1] Hatanaka, Y. and Sadakane, Y., Curr. Topics Med. Chem. ***2002****, 2, 271-288.*

P080

HIGH-LEVEL EXPRESSION IN PICHIA PASTORIS AND CHARACTERISATION OF BIOLOGICALLY ACTIVE ^{15}N-LABELED HUMAN CHORIONIC GONADOTROPIN

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Human chorionic gonadotropin (hCG) is a heterodimeric glycoprotein hormone involved in the maintenance of the corpus luteum during the first trimester of pregnancy. Usually, hCG is isolated from urine of pregnant women. Biologically active hCG has been successfully expressed in the methylotrophic yeast Pichia pastoris (phCG) (Gadkari et al., 2003) whereby the glycosylation pattern comprises $Man_8GlcNAc_2$ to $Man_{11}GlcNAc_2$ N-glycans, which are eventually phosphorylated (Blanchard et al., 2005). Being interested in the structure of hCG in solution, applying NMR spectroscopy, we have focused here on the expression in P. pastoris and characterisation of biologically active ^{15}N-labeled phCG.

The fermentation of phCG was performed in a 3-liter bioreactor maintained at 28 °C, in which 5 to 10 g/l of $^{15}NH_4Cl$ were fed every 24 h. At the end of the batch phase, just before methanol induction, the wet cell weight reached 109 g/l. Such a high cell density has not been reported earlier for recombinant glycoprotein hormones. Finally, about 30 mg of ^{15}N-phCG could be purified from the 3-liter culture supernatant. The protein was characterised by NMR spectroscopy, MALDI-TOF mass spectrometry, Circular Dichroism, RIA and RRA.

P081

DESIGN OF MECHANISM-BASED PROBE FOR NEURAMINIDASE TO CAPTURE INFLUENZA VIRUSES

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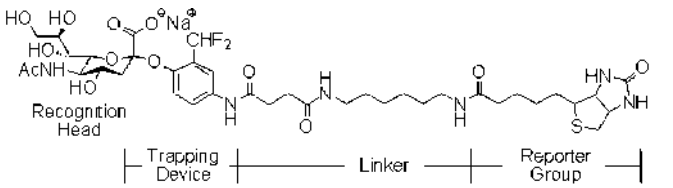
A mechanism-based probe for the detection and inhibition of influenza virus neuraminidases has been designed and synthesized. The probe structurally contains four fragments, a sialic acid for recognition, a latent quinone methide for trapping device, a linker, and a biotin as reporter for detection and separation. Once sialic acid released by the action of neuraminidase, the enzyme can be covalent modification by the resulting highly reactive quinone methide. In our study, the biotin labeled *Arthrobacter ureafaciens* neuraminidases were observed on western blotting. For virus-capturing experiment, it could be used to attach the probe to the microtiterplates through avidin-biotin interactions and the ELISA assay successfully demonstrated that influenza could be selectively captured with this probe. This novel approach offers opportunities to rapid screening of antibodies against influenza virus and development of sensitive and rapid diagnostic methods.



P082

ANTIMICROBIAL POTENTIAL OF ARABINOGALACTAN PROTEINS (AGPS) LIKE MOLECULES FROM *NERIUM ODORUM* LEAVES

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Arabinogalactan proteins (AGPs) are a class of structurally complex, heterogeneous glycol-conjugates of plant origin and found to have potential use as immunomodulator,

antimicrobial, anticancerous and in somatic embryogenesis induction. Keeping the multifarious and the therapeutic application of AGPs in mind, an attempt has been made to the study of arabinogalactan protein from *Nerium odorum* an Indian medicinal plant of apocyanaceae family. Arabinogalactan proteins (AGP) was isolated and purify from the leaves of *Nerium odorum* and quantified with β-glucosyl Yariv reagent that is specifically bind to AGP like molecule followed by gel filtration chromatography. Protein and carbohydrate concentration in the sample was determined using BCA reagent and Ferricyanide assay respectively. The purified AGP preparation was separated on 12% SDS PAGE followed by Coommassie blue, Yariv reagent and alcian blue staining. The antifungal activity of purified AGP tested against *Aspergillus niger, Aspergillus fumigatus, Aspergillus flavus, Candida albicans*, *Fusarium oxysporum* and *Cryptococcus neoformans* using disc diffusion assay. Antibacterial activity of the isolated AGPs was evaluated against various bacterial species *Staphylococcus aureus, Pseudomonas putida, Bacillus substillis,* and *E. coli.* The AGP preparation from *Nerium odorum* had significant antifungal and antibacterial activity. The present study suggests that AGP could be good antimicrobial agents due to its high water solubility and less cytotoxic nature. The finding will be presented and discuss.

P083
CHARACTERIZATION OF SINGLE CHAIN ANTIBODIES (SCFVS) AGAINST VARIOUS TYPES OF CARBOHYDRATE EPITOPES

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Phage-display technology has successfully been used to produce human scFvs against peptides/proteins without immunizing animals. The aim of this project is to build up a set of scFvs against a variety of carbohydrate epitopes using this technology. Due to difficulties in immobilization of carbohydrate antigens onto plastic plates, however, the same procedures used for protein antigens cannot be readily applied. Thus, methodologies have been developed for carbohydrate-based antigen, which resulted in isolation of first series of scFvs against various types of carbohydrate epitopes.
A phage-display library representing over 10^{11} independent human scFvs was subjected to four rounds of panning against synthetic glycolipids bearing various carbohydrate structures. Specific antibodies were screened and identified by ELISA.
Positive clones were analyzed by PCR amplification of scFv inserts followed by DNA sequence analysis. scFvs were prepared from periplasmic fractions of host *E.coli*. Carbohydrate specificity and affinity of both scFvs and their phage-displayed counterparts were determined by ELISA, TLC-overlay assay, and surface plasmon resornance analyses using neoglycolipids/glycoproteins.
Of 864 colonies screened, 25 positive clones against trimannose-dipalmitoylphosphatidyl-ethanolamine were obtained whereas only 5 positive clones against Lewis-related carbohydrates were identified. In contrast, of 96 colonies screened, 13 positive clones against T antigen-hexa(ethyleneglycol)-lipid have been isolated. Positive clones exhibited specificity for carbohydrates but not for lipids.
This study has demonstrated a potential use of phage-display strategy in isolating human scFvs against various carbohydrate antigens, but suggests that the outcome of screening appears to be dependent on types of carbohydrate antigens and/or ways of carbohydrate antigen presentation to scFvs.

P084
INTRACELLULAR VISUALIZATION OF VIP36 OLIGOMERIZATION USING FRET ANALYSIS

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Soluble secretary proteins are packaged into transport vesicles with the aids of transmembrane cargo receptors. Cargo receptors participate in not only packaging but also trafficking and sorting of cargoes. L-type lectin family acts as cargo receptors, which recognize carbohydrate moieties as transport signals of glycoproteins. Transport mechanism of cargo receptor is thought to be based on the 'catch and release' mechanism, by which cargo receptors capture cargoes in an in

intracellular compartment and release in another compartment. However, the regulatory mechanism of sugar-binding activity of cargo receptors is not fully understood. Our previous work using biochemical analysis showed that one of cargo receptors VIP36 oligomerizes and increases the avidity for carbohydrates under acidic condition. This suggests that VIP36 catches cargoes in the trans-Golgi network (pH6.0) and releases in the ER (pH7.2) or on the cell surface (pH7.4). However, this hypothetical model has not been confirmed within the cells. To examine this model intracellularly, fluorescent resonance energy transfer (FRET) technique was applied to VIP36. VIP36 fused with cyan fluorescence protein (CFP) and yellow fluorescent protein (YFP) at its C-terminus were co-expressed in CHO cells and FRET was quantified by acceptor bleaching method. The cells expressing both VIP36-CFP and VIP36-YFP demonstrated the interaction of these proteins at FRET efficiency approximately 22% indicating that VIP36 oligomerizes within the cells. In this work, we demonstrated self-association of VIP36 intracellularly, which had already been shown in vitro. Moreover FRET could be useful as a new approach to investigate molecular interaction in lectin research.

Glycoinformatics

P085
THE ANIMAL SIALYLTRANSFERASES AND SIALYLTRANSFERASE-RELATED GENES: A PHYLOGENETIC APPROACH.

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The animal sialyltransferases are Golgi type II transmembrane glycosyltransferases. Twenty distinct sialyltransferases genes have been identified in both human and murine genomes. These enzymes catalyze transfer of sialic acid from CMP-Neu5Ac to the glycan moiety of glycoconjugates. Despite low overall sequence identities, they share four conserved peptide motifs (L, S, motif III and VS) that are hallmarks for sialyltransferase identification. Genome sequencing programs offer a new route into understanding multigene families both within a single species and across different species. We have identified 155 new putative genes in 25 animal species based on two lines of evidence: (i) sequence comparisons and (ii) exon-intron organization of the genes. An ortholog to the ancestor present before the split of ST6Gal I and II subfamilies was detected in arthropods. An ortholog to the ancestor present before the split of ST6GalNAc III, IV, V and VI subfamilies was detected in sea urchin. An ortholog to the ancestor present before the split of ST3Gal I and II subfamilies was detected in ciona and an ortholog to the ancestor of all the ST8Sia was detected in amphioxus. Therefore, single examples of the four families (ST3Gal, ST6Gal, ST6GalNAc and ST8Sia) have appeared in invertebrates, earlier than previously thought, whereas the four families were all detected in vertebrates suggesting a common genetic origin by successive duplications of an ancestral gene, followed by divergent evolution. Finally, we propose predictions on these invertebrate sialyltransferase-related activities that are not yet demonstrated.

P086
ALPHA-(1->6)-GLYCOSIDIC LINKAGE AS A CONFORMATIONAL ENTROPIC REGULATOR IN PERIPLASMIC OSMOREGULATED CYCLIC GLUCAN

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Molecular order-disordering or conformational entropy is an essential determinant in the physico-chemical properties of macromolecules. In this paper, we investigated the alpha-(1->6)-glycosidic linkage as a conformational entropic regulator of microbial cyclooligosaccharide, which is a kind of cell-associated osmoregulated periplasmic glucans (OPG). Molecular dynamics (MD) simulations were performed to explain conformational effect of α-(1->6)-glycosidic linkage upon cyclic OPG produced by *Xanthomonas campestris* pv. *citri.* The MD simulations were performed using the CHARMM 28b2 program in the isothermal-isobaric ensemble = 1 bar, T = 298 K). The vibrational entropy $T\Delta S_{vib}$ was calculated to be reduced by 11.92 kJmol^{-1} if the α-(1->6)-glycosidic linkage was introduced to β-(1->2)-cyclic OPG at 298K. However, rotational motions of adjacent water were increased from the rotational correlation time measurement. We suggest that a single α-(1->6)-glycosidic linkage in cyclic OPG functions as a novel entropic regulator, which reduces the conformational entropy of cyclic OPG and increases the motional entropy of solvent water molecules. Our goal is to suggest the presence of an entropic regulator as a form of a specific linkage pattern in the carbohydrate folding and to clarify the relationship between the conformational entropy of the carbohydrate molecules and the biological function at an atomic level throughout the molecular dynamics simulations.

P087

HYDROPHOBIC METHYL CAP OF FLAVONOID/β-CYCLODEXTRIN INCLUSION COMPLEX AS A DETERMINANT FACTOR FOR AQUEOUS SOLUBILITY

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The flavonoids are members of natural compounds produced by the green plant cells. They are potential medical applicants for hypertension, cancer, inflammation, and cardiovascular disease. But easy-use of the flavonoid has been regarded as a long-standing problem for many people because its aqueous solubility is very low. Cyclodextrins (CDs) and their derivatives are one of the most important solubilizing agents for various drugs, food additives, and other hydrophobic molecules including various flavonoids. In view of limited amount of the structural information available, computational methods can provide a useful means to develop theoretical model for aqueous solubility of flavonoid/β-CD complexes. This study describes the thermodynamic and structural basis for the flavonoid/β-CD inclusion complexes with regard to the origin of solubility difference between hesperetin/β-CD and naringenin/β-CD complexes. The aqueous solubility of each flavonoid/β-CD complex could be characterized by complex-water interaction not by flavonoid-CD interaction during molecular dynamics simulations. The radial distribution of water around each inclusion complex elucidated the difference of an experimentally observed solubility of each flavonoid/β-CD complex. The analyzed results suggested that a bulky hydrophobic moiety (-OCH_3) of *B*-ring of hesperetin nearby primary rim of β-CD was responsible for lower aqueous solubility of the hesperetin/β-CD complex. In this respect, the geometric design of docked conformation for the inclusion complex would be highly recommended to gain a desirable aqueous solubility of flavonoid compounds based on inclusion complex

P088

IN SILICO INVESTIGATION OF CONFORMATIONAL MAP OF THE SERIES OF GLUCO-, GALACTO- AND MANNO-DISACCHARIDES VIA 1-1, 1-2, 1-3, 1-4, and 1-6 GLYCOSIDIC LINKAGES

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Carbohydrate conformations can be basically characterized by the glycosidic linkages between two monosaccharide units. Thus establishment of the conformational map of disaccharides is an important starting point of structural study on the carbohydrates or glycoconjugates. In practice it is difficult to obtain a crystal or solution structure of the carbohydrate including disaccharides by using an X-ray or NMR spectroscopic method, due to the highly flexible rotational motion via glycosidic linkages. This study comprises molecular dynamics (MD) simulations on the conformational status of the series of α-and β- gluco-, galacto- and mannopyranosic homodisaccharide with possible 5-glycosidic linkages, 1-1, 1-2, 1-3, 1-4, and 1-6 linkages. In total, conformational maps on the 57-glycosidic linkage combinations were established. The long-time MD simulations in explicit TIP3P water were performed with CHARMM program under *NPT* conditions. Each disaccharide was modeled and parameterized with the specific carbohydrate solution force field (CSFF) of the CHARMM. The major concerns in this study were the comparison between the X-ray or NMR-determined ϕ and ψ dihedral angles and those determined by the MD simulations. The second subject was the analysis on the inter- and intra-hydrogen-bonding pattern of each disaccharide in aqueous solvent. The two-dimensional population density maps for the ϕ and ψ dihedral angles during MD simulations indicated that the computational approach was much reliable as compared with experimentally determined dihedral conformations. In our knowledge, that is the first systematic approach to establish a conformational status of the carbohydrates with the long-time MD simulations in explicit water.

P089

PREDICTION FOR THE AQUEOUS SOLUBILITY OF ORGANIC COMPOUNDS COMPLEXED WITH α-, β-, or γ-CYCLODEXTRIN BASED ON MONTE CARLO DOCKING SIMULATIONS

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Aqueous solubility is an important physical property that influences the release or transport of drugs in the human body. Many poorly insoluble drugs have been physically or chemically modified to enhance their aqueous solubility and

availability. Cyclodextrins (CDs) and their derivatives are common solubilizing agents for various drugs, food additives, and other hydrophobic small molecules. A-type phase-solubility profiles are obtained when the solubility of the guest compounds increases with increasing host cyclodextrin concentration. Under A_L-type phase-solubility diagram, aqueous solubility of guest compounds complexed with cyclodextrin can be described with the slope (*S*) of the solubility curve and the intrinsic solubility (S_0) of the guests. To improve the commercial applications of solubility enhancement by CDs, accurate computational method to estimate aqueous solubility of inclusion complex is highly desirable. The present paper describes the theoretical model of solubility for the A_L-type guest/CD inclusion complexes in order to establish a precise prediction method for the aqueous solubility of inclusion complexes. A Monte Carlo (MC) docking method was introduced in order to predict the aqueous solubility of inclusion complexes composed of small organic compounds and various cyclodextrins. The slope (*S*) of the A_L-type phase solubility curve was accurately predicted by a combination of the interaction energy and nonpolar solvation free energy for each of the docked complexes. The regression equation for *S,* the slope of the phase solubility curve gives a fine correlation coefficient, r^2, of 0.913 and standard error of 0.028 for the 63 organic compounds complexed with cyclodextrins.

P090
DEVELOPMENT OF THE DATABASE FOR STRUCTURAL GLYCOMICS

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Structural glycomics is playing a fundamental role on the analysis of glycan functions (i.e. , functional glycomics) along with the development of a high throughput carbohydrate sequencing technology such as mass spectrometry. However there are few bioinformatics resources for glycomics/glycoproteomic. We developed a database for structural glycomics, “CabosDB”. CabosDB consists of oligosaccharide database, lectin database and glycoprotein database. The oligosaccharide database contains oligosaccharide structures described by carbohydrate sequence markup language (CabosML[1]), their mass spectra and the experimental conditions. User can search for any oligosaccharide in the database using a glycan editor. Mass spectra were automatically interpreted by comparing to theoretical values of the oligosaccharide fragment structures. Oligosaccharide database has a web service for rapid identification of oligosaccharide structures of mass spectra. User can determine the structure of analyte using interface software that connects Shimadzu/Kratos AXIMA QIT with our web service. Lectin database contains lectin molecules and lectin map. Lectin molecules include family, CRD, amino acid sequences, function, tertiary structures and binding sites. Lectin map includes affinity constants between lectins and oligosaccharides measured by frontal affinity chromatography. Glycoprotein database contains glycoprotein sequences and glycan binding sites identified by IGOT method. CabosDB has a web interface to access their information including graphical representation of data (glycan binding sites and affinity constants etc.).

1: Kikuchi N, Kameyama A, Nakaya S, Ito H, Sato T, Shikanai T, Takahashi Y,
Narimatsu H. The carbohydrate sequence markup language (CabosML): an XML description of carbohydrate structures. Bioinformatics. 2005 Apr 15;21(8):1717-8.

P091
KEM-C IS A USEFUL TOOL WHICH OUTPUTS GLYCOSYLTRANSFERASES REQUIRED FOR THE SYNTHESIS OF A DESIRED PRODUCT AND A REACTION PATHWAY, BASED ON INPUT INFORMATION ON THE STRUCTURE OF A DESIRED PRODUCT.

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Glycosylation has been recognized as the most common post-translational modification in vivo. Although oligosaccharides on glycoconjugates play vital roles in biological processes, influencing the stability and conformation of proteins, intra- and inter-cell signaling, and binding specificity for other biomolecules, their functions are little understood at the molecular level. However, the analysis of the structure and interaction of oligosaccharides requires a large variety of structurally defined oligosaccharides. We reported a novel method for the efficient construction of a glycopeptide library which easily converted to an oligosaccharide library, using human recombinant glycosyltransferases(GTs). To synthesize oligosaccharides using GTs, a lot of knowledge about reaction pathway and experimental conditions for each GTs is required.
We have developed a tool, named KEM-C, to search the database. Based on input information on the structure of a desired product by user, it identifies and lists a set of GTs and their donors in a series of enzymatically catalyzed reaction of a desired product. It enables us to easily obtain a GTs list, as well as the reaction pathway, required for a glycan synthesis.

This work was supported by the New Energy and Industrial Technology Development Organization (NEDO).

P092
A NOVEL APPLICATION OF LECTIN MAP BASED ON PATTERN RECOGNITION FOR IDENTIFYING OLIGOSACCHARIDE STRUCTURES

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The completion of the 'Lectin Map', which provides comprehensive, quantitative data on affinities between 100 lectins and 100 glycans measured by high-throughput frontal affinity chromatography technology is expected to contribute greatly as an essential and fundamental resource for glycoproteomics. Our preliminary cluster analysis of a currently developing Lectin Map suggested that affinity patterning could classify the structural features of oligosaccharides satisfactorily. Accordingly, we developed two novel methods based on machine learning and pattern recognition to extract useful lectins from the Lectin Map for identifying oligosaccharide structures. One is a *discriminating* method, which determines appropriate lectins to discriminate each one of the denoted structural features of interest using Support Vector Machine, a powerful binary classifier. The other is a c*ombination* method to search for an optimized set of lectins, which can distinguish each member of the oligosaccharide library by comprehensive comparing manner. To validate the utility of these methods, the affinities between 52 lectins and 44 N-glycans were examined. The *discriminating* method determined some lectins as identifiers for certain structural categories of N-glycan (*e.g.,* high-mannnose-type, complex-type, bisecting-GlcNAc, fucosylated) successfully. Further, combining these lectins could also separate more complicated structures. On the other hands, combination of a relatively small number of lectins (*e.g.,* <5) was found to be effective for discrimination of the 44 N-glycan structures by the *combination* method. Taken together, our methods proved to be highly promising for glycan profiling of even more diverse oligosaccharides.

P093
NEW DATABASE OF BACTERIAL CARBOHYDRATE STRUCTURES

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CSDB, a database containing data on natural carbohydrates with known primary structure, has been developed. In addition to the structure and bibliography, each record in the CSDB contains the abstract of the publication, data on the carbohydrate source, methods of structure elucidation, information on the availability of spectral data and assignment of NMR spectra when available, data on conformation, biological activity, chemical and enzymatic synthesis, biosynthesis, genetics and other related data. The key feature is the possibility to search within the database using as parameter a fragment(s) of the structure or indexed tags, including carbohydrate source, keywords and bibliography.
Currently, the CSDB contains about 7500 records on bacterial carbohydrates, including the corresponding part of CarbBank, a database developed at the end of XXth century (about 3000 records on structures reported before 1995). This coverage is approaching the total number of bacterial carbohydrate structures ever reported. Data from both literature and CarbBank have been checked for consistency prior to the upload and corrected when necessary.
The CSDB interface includes the web-based user part, web-based administrator part and programming gateways for automated data interchange. A possibility is provided for cross-referring to other databases containing information on natural carbohydrates. The CSDB is available at http://www.glyco.ac.ru for free usage and validated user data submission.
This work was performed within the framework of the ISTC Partner Project #1197, supported by the CTR Program of the US Department of Defense (ISTC Partner), and the RFBR grant 05-07-90099.

P094
MILK OLIGOSACCHARIDES OF THE CANOIDEA (CARNIVORA)

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The milk of eutherian mammals usually contains carbohydrate, the concentration of which varies between different species. The milk of several members of the superfamily Canoidea of the Carnivora differs from that of other eutherians in that it contains higher concentrations of oligosaccharides than lactose. Although more than 90% of the carbohydrate of dog (Canidae) mature milk is lactose, this is not the case for milk of the white nosed coati (Procyonidae), mink (Mustelidae), phocid seals (Phocidae), bears (Ursidae) and the giant panda (Ailuropodae); in some of these species the milk contains only traces of lactose and much larger amounts of oligosaccharides. The neutral and acidic milk

oligosaccharides have been characterized in these Canoidea species. All the oligosaccharides have lactose, lacto-N-neotetraose, lacto-N-neohexaose or para lacto-N-neohexaose as core units and are type 2. Some of the milk oligosaccharides of bears, giant panda, mink and coati have alpha-Gal at their non-reducing ends whereas those of seals do not. Some of the milk oligosaccharides of mink, coati and seals have H antigen at their non reducing ends. By contrast, some of the bear milk oligosaccharides contain ABH antigen at their non – reducing ends and the milk oligosaccharides of other species of Canoidea do not contain A or B antigens. The bear milk oligosaccharides contain Lewis x. Most of the acidic milk oligosaccharides of bears, mink and harbour seal were found to have a alpha (2-6) linked Neu5Ac residues attached to a lacto-N-neohexaose unit.

P095
ANALYSIS OF PROTEIN-CARBOHYDRATE INTERACTIONS CONTAINED IN THE PDB

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Carbohydrate-protein interactions are implicated in a variety of cell-cell and cell-matrix recognition events, ranging from fertilisation, cellular differentiation and development to pathological situations like inflammation, viral and bacterial infections, immune response and metastasis. These events require a specific recognition of different carbohydrate structures by carbohydrate-binding proteins, the lectins.
To be able to understand these processes in detail, it is often indispensable to know the 3D structures of the protein-carbohydrate-complexes. The largest publicly available source of such 3D structures is the Protein Data Bank (PDB).
Data are analysed using the GlyVicinity software, which can be accessed at the URL: www.glycosciences.de/glyvicinity/
In the spatial vicinity of carbohydrates, polar amino acids are over-represented, while – with the exception of aromatic residues – non-polar amino acids are under-represented. Around different carbohydrate residues, like β-D-Gal*p*, α-D-Neu*p*5Ac, uronic acids or sulphated residues, different patterns of amino acids are observed, which results from the fact that most carbohydrate recognition sites of proteins are specific for certain carbohydrates.
Analysis of the atoms involved in the protein-carbohydrate interactions reveals information on the kind of interactions. For β-D-Gal*p* residues, stacking interactions to aromatic residues, especially Trp, are of particular importance. In the vicinity of the negatively charged carbohydrates α-D-Neu*p*5Ac and uronic acids, Trp is also over-represented, but these residues mainly form electrostatic interactions to amino acids. Around the also negatively charged sulphated carbohydrates, positively charged amino acids (Arg, Lys, His) are more highly over-represented than around the α-D-Neu*p*5Ac and uronic acids.

Glycotherapeutics and glycoindustries

P096
STRUCTURAL ANALYSIS OF THE N-GLYCOSYLATION OF RECOMBINANT HUMAN ACID α-GLUCOSIDASE EXPRESSED IN MILK OF TRANSGENIC RABBITS

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The lack of or a major deficiency in acid α-glucosidase (1,4-α-D-glucan glucohydrolase; EC 3.2.1.3/20) is characteristic for the glycogen storage disease, known as Pompe disease. Several gene mutations in the acid α-glucosidase encoding gene have been observed so far. Treatment of the disease is performed by intravenous injection of recombinant forms of the enzyme. Specific attention has to be paid to the generated glycosylation patterns when focusing on recombinant approaches to produce the enzyme. Here, human acid α-glucosidase was expressed in the mammary gland of transgenic rabbits and isolated from the milk. Via peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F treatment, the N-linked glycans of recombinant human acid α-glucosidase (rhAGLU) were released. Fractionation and purification of the glycan pool into individual components was done by a combination of anion-exchange, normal-phase and SNA lectin-affinity chromatography. Analysis by 500 MHz one-dimensional and two-dimensional (TOCSY) ^{1}H-NMR spectroscopy, combined with 125.759 MHz two-dimensional ^{31}P-NMR RESED spectroscopy, and MALDI-TOF mass spectrometry, yielded the structures of the N-glycans. rhAGLU has a glycosylation pattern consisting of neutral, mono- and di-charged N-glycans. The alternative glycosylation pathway is suggested by the finding of a major amount of a $Man_8GlcNAc_2$-isomer missing Man on the Man(α1-3)Man(β1-4) arm. Partially fucosylated complex-type structures, containing (α2-6)-linked Neu5Ac, were found, as well as two rare hybrid-type structures. A fraction of the oligomannose-type glycans contained phospho-diester-bridged *N*-acetylglucosamines. Profiling and exo-glycosidase studies confirmed the structures found by NMR and the absence of terminal phosphate groups.

P097
REGULATION OF CELL PROLIFERATION AND SPECIFIC GENE EXPRESSION IN CHONDROCYTES BY ADDITION OF EXOGENOUS CARTILAGE PROTEOGLYCAN

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Proteoglycan (PG) is an important molecule affecting physiological roles such as cell attachment, migration, differentiation, and morphogenesis. Although studies have been done on mechanical role of cartilage PG, its roles on proliferation and differentiation of chondrocytes remain unclear. In this study, we have examined the effect of exogenous cartilage PG on cell proliferation and gene expression in chondrocytes in two-dimensional (2-D) and three-dimensional (3-D) culture.
In 2-D culture, rabbit articular cartilage chondrocytes were grown on culture dishs coated with PG from salmon nasal cartilage. In 3-D culture, chondrocytes were cultured in atelocollagen gel mixed with PG. After various periods of culturing, the number of cells were counted by hemocytometer. Gene expressions of type I/II/X collagen and aggrecan were quantified by real-time PCR.
In both cultures, proliferation of chondrocytes treated with low concentration PG was significantly decreased compared to that of control cells without PG treatment. However, high concentration PG significantly enhanced proliferation of chondrocytes. In 2-D culture, gene expressions of type X collagen and aggrecan in chondrocytes treated with low concentration PG were increased. By contrast, gene expressions of type II/X collagen in chodrocytes treated with high concentration PG were decreased. In 3-D culture, gene expression of aggrecan in chondrocytes treated with PG were increased.
The treatment with low and high concentration PG oppositely affected the chondrocyte proliferation, suggesting that the different mechanisms of cell proliferation were induced by PG. Elucidation of these mechanisms may lead to discovery of a new therapeutic method for chondral lesion.

P098
EFFICIENT CONVERGENT SYNTHESIS OF LINEAR SELECTIVELY SULFATED TETRA-, HEXA- AND OCTASACCHARIDE FUCOIDAN FRAGMENTS

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Brown seaweed polysaccharides fucoidans inhibit microbial adhesion, angiogenesis, P- and L-selectin mediated inflammation, blood coagulation. In this communication we report on the efficient blockwise highly stereoselective synthesis of linear fucoidan fragments α-L-Fuc*p*-(1→3)-[α-L-Fuc*p*-(1→3)]$_n$-α-L-Fuc*p*-OPr (n = 2, 4, 6) and α-L-Fuc*p*-(1→4)-[α-L-Fuc*p*-(1→3)-α-L-Fuc*p*-(1→4)]$_n$-α-L-Fuc*p*-OPr (n = 1, 2) bearing sulfonato-groups at O-2 of fucose residues. We use [2+2], [2+4] and [4+4] carbohydrate chain assembling strategy for the synthesis of tetra-, hexa- and octasaccharides, respectively. These oligosaccharides are model compounds to assess fragments of fucoidan chains responsible for their biological activity.

P099
4-METHYLUMBELLIFERONE, A HYALURONAN SYNTHASE SUPPRESSOR, ENHANCES THE ANTICANCER ACTIVITY OF GEMCITABINE IN HUMAN PANCREATIC CANCER CELLS

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Introduction
Hyaluronan (HA) is a ubiquitous, major component of the pericellular matrix and is necessary for various physiological processes. It plays a very important role in biological barriers. We hypothesized that MU-mediated inhibition of HA synthesis and pericellular HA matrix formation would increase the efficacy of anticancer drugs.
Experimental approaches
We chose a human pancreatic cancer cell line as our cancer cells and gemcitabine as our anticancer agent. *In vitro*, we investigated whether MU inhibited HA synthesis of pancreatic cancer cells and formation of the pericellular HA matrix. We then investigated whether reduction of the pericellular HA matrix influenced the anticancer activity of gemcitabine. *In vivo*, we investigated whether administration of both MU and gemcitabine influenced primary tumor growth and inhibited liver metastasis as compared with administration of gemcitabine alone in mice that had been implanted with pancreatic cancer cells and also had severe combined immunodeficiency disease (SCID).
Result
We demonstrated *in vitro* that MU inhibits HA synthesis and formation of the pericellular HA matrix, in human KP1-NL pancreatic cancer cells. AlamarBlue assay revealed that the anticancer effect of gemcitabine in KP1-NL cells was increased by pretreatment with MU. *In vivo* simultaneous administration of MU and gemcitabine to tumor-bearing SCID mice decreased the size of the primary and metastatic tumors more than did gemcitabine alone.
Conclusion

These data strongly suggest that a combination of MU and gemcitabine is effective against human pancreatic cancer cells. MU may have potential as a chemosensitizer and may provide us with a new anticancer strategy.

Physico-chemistry and membrane behavior of glycolipids

P100
GM3 AGGREGATES WITH DIFFERENT COUNTERIONS: FROM CONNECTED TO DISCONNECTED STRUCTURES.

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We report on Small Angle X-ray Scattering (SAXS) experiments performed on the ganglioside GM3, in water solution, with Na^+, Rb^+ and Ca^{++} counterions. Experiments were carried out with no added salt, in the concentration range from very dilute to semidilute (0.1-15 per cent bw), and in the range of temperature from 15°C to 60°C. On the local scale, a bilayer-like cross section is preserved all over the investigated range of concentration and for the three counterion substitution However, the structure adopted on the mesoscopic scale does depend on counterion. *a)* In the case of Na^+, the system adopts a connected direct structure (also called lace-like structure) nearly all over the investigated range of concentration. It is striking that the connected structure can be adopted even at very low volume fractions (of the order of per cent). *b)* In the case of Rb^+, the system adopts a disconnected structure and becomes hexagonally ordered above 3 per cent concentration. *c)* In the case of GM3-Ca^{++} a strong coordination between GM3 aggregates occurs and results in the formation of nano-flocks. These structural results experimentally highlight the ability of gangliosides, in their physiological form, to widely coordinate their environment. Moreover they reflect a higher bending rigidity of the aggregates of GM3-Rb^+ (non-physiological) as compared to those of GM3-Na^+ (physiological). Finally, the well known ability of calcium in promoting complexation can be recognized.

P101
BENDING RIGIDITY OF LARGE UNILAMELLAR LIPID VESICLES: INFLUENCE OF THE PRESENCE AND DISPOSITION OF GANGLIOSIDES.

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We apply the dynamic laser light scattering technique to investigate the influence of added gangliosides on the characteristic times of thermally induced shape fluctuation of phospholipid large unilamellar vesicles (LUVs). The experimental results show that the addition of gangliosides, even in small amount (lower than 2% mole ratio), in DMPC vesicles induces a change in the membrane bending rigidity, strongly depending on its disposition within the bilayer. In fact, quite unexpectedly, the *symmetric* (in both leaflets) or *asymmetric* (only in the outer leaflet) disposition of gangliosides has opposite effects on bilayer dynamics. The *symmetric* disposition causes a "hardening" of the membrane (similar to the one tested for cholesterol, but added in much higher amount), while the *asymmetric* disposition "softens" the membrane. Those results reinforce the hypothesis that the local structure of the bilayer (both in the transbilayer and in the lateral directions) is important in determining the overall dynamics of the vesicle. They show how the physiological disposition of gangliosides in the outer leaflet of real cells also can have a mechanical implication, facilitating outward (protrusion) or inward (invagination) motions.

P102
CHANGE OF FORSSMAN GLYCOLIPID EXPRESSIONIN MOUSE ES AND F9 CELLS

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Cell surface glycoconjugates are considered to behave as important key molecules in cell-cell and cell recognition. The change of the diverse structures are often associated with differentiation event. Although Forssman glycolipid (FG) has been reported to be one of the heterophile antigen, this expression of the carbohydrate antigen is also remarkably changes during differentiation. To understand the expression and the molecular mechanism, FG expression was investigated in mouse ES and F9 cells. Methods and Materials; Flow cytometric and fluorescein microscopic analyses were done by using specific antibodies to FG and Gb4. Distribution of FG and Gb4during the differentiation were investigated by the preparation of raft from both undifferentiated and differentiated F9 cells Results and Discussions; Both cells showed the high expression level in undifferentiated stage. However, this high level expression was decreased with differentiation. mRNA level of FG synthtase exhibited the different expression pattern to that of cell surface carbohydrate antigen. This evidence was also confirmed by the glycolipid analysis on TLC and TLC-immunostaining. Fluorescein microscopic analyses by FG specific antibody indicated that FG is located not only at the

cell surface but also in the cell. Taken together above, the FG expression did not correlated the mRNA of FG synthtase, suggesting that there might be other factors involved in the regulation of FG expression. Studies are in progress to address the mechanism of the decreased FG expression and biological consequences of the decline of FG expression containing the preparation of the microdomain lipid fraction from F9 cells

P103
TRYPTOPHAN INVOLVEMENT IN THE GLYCOLIPID TRANSFER PROTEIN ACTIVITY

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The glycolipid transfer protein (GLTP) specifically transfers glycolipids with a beta-linkage between ceramide or diacylglycerol and the sugar moiety. With site-directed mutagenesis we constructed single tryptophan mutants where two of the three tryptophan (W) of wild-type human GLTP were substituted with phenylalanine (F) and named W85 GLTP (W96F&W142F), W96 GLTP (W85F&W142F) and W142 GLTP (W85F&W96F) accordingly. Binding of the various proteins to lipid vesicles was investigated by lifetime and steady-state fluorescence techniques and secondary structure with circular dichroism. Wild-type GLTP and W96 GLTP were both able to transfer galactosylceramide, but the two variants W85 GLTP and W142 GLTP did not show any glycolipid transfer activity, indicating that the W in position 96 is crucial for transfer activity. Tryptophan fluorescence emission showed a blue shift of the maximal emission wavelength upon interaction of glycolipid containing vesicle with wild-type GLTP and W96 GLTP, while no blue shift was recorded for the protein variants W85 GLTP and W142 GLTP. The quantum yield of W emission was highest for the W96 GLTP protein whereas W85 GLTP, W142 GLTP and wild-type GLTP showed a lower and almost similar quantum yield. The lifetime and anisotropy decay of the different W mutants also changed upon binding to different vesicles. Again wild-type GLTP and W96 GLTP showed similar behavior in the presence of vesicles containing glycolipids. Taken together, our data show that the W96 is involved not only in the activity of the protein but also in the interaction between the protein and glycolipid containing membranes.

P104
GLYCOLIPIDS FROM *Leishmania (Viannia) braziliensis* PROMASTIGOTES LOCALIZED IN DETERGENT-RESISTENT MEMBRANE DOMAINS

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Recent works have suggested that (glyco)lipid microdomains are present in *Leishmania* membrane. These specialized membrane domains are termed detergent-resistant membranes (DRMs), based on differential properties when extracted with Triton X-100 at different temperatures. The plasma membrane domains from *Leishmania (Viannia) braziliensis* promastigotes insoluble with 1% of Triton X-100 at 4 C were fractionated by ultracentrifugation in sucrose gradient, and the presence of DRMs was confirmed. *Leishmania (Viannia) braziliensis* promastigote DRMs are composed by glycoinositolphospholipids (GIPLs), inositol phosphorylceramide (IPC), phosphatidylinositol, phosphatidylethanolamine and ergosterol. On the other hand, Triton X-100 soluble fraction presented: phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, IPC, ergosterol and *lyso*-phosphatidylinositol. By HPTLC immunostaining using mAb SST-1, it was showed that 85% of GIPLs are present in DRMs, and by immunoelectronmicroscopy it is clear that components reactive with SST-1 are located in patches along the parasite surface. No difference in the GIPLs pattern was observed by HPTLC between the fractions soluble and insoluble with Triton X-100 at 4 C. Furthermore, fatty acid composition analyzed by GC/MS showed in DRMs the presence of GIPLs containing an alkylacylglycerol, presenting mainly saturated acyl and alkyl chains. It was also detected in DRMs ergosterol, IPC containing saturated fatty acid, phosphatidylinositol with at least one saturated acyl chain, and phosphatidylethanolamine presenting predominantly oleic acid. The role of the DRMs in *Leishmania* pathogenicity is still unclear, it is suggestive that the preferential distribution of GIPLs and IPC in DRMs may be related to the parasite infectivity processes.
Supported by CAPES, CNPq and FAPESP

Molecular mechanisms of carbohydrate-carbohydrate & protein interaction

P105
MOLECULAR CHAPERONE BIP BINDS TO CARGO RECEPTOR VIP36 CONSTITUTIVELY

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VIP36 is an intracellular lectin distributed from the ER to the Golgi apparatus, and is thought to act as a cargo receptor to

involve in the transport and sorting of glycoproteins. Crosslinking and subsequent immunoprecipitation of VIP36 from 293 cells expressing Myc-tagged VIP36 showed that the presence of a ~80 kD protein was coprecipitated with VIP36 on a SDS-polyacrylamide gel. The protein was also copurified to the same extent with VIP36 mutant that has a defect in lectin activity, indicating that the interaction between VIP36 and ~80 kD protein is carbohydrate-independent. We performed the same immunoprecipitation experiment on a large scale, and identified a coprecipitated protein by LC/MS/MS analysis as immunoglobulin binding protein (BiP), which is a main protein of Hsp70 chaperone family. A pulse-chase experiment using [^{35}S]methionine/cysteine revealed that the interaction of VIP36 and BiP is stable. The interaction of these proteins was further confirmed *in vitro* by surface plasmon resonance analysis using recombinant proteins expressed *E. coli* cells. We demonstrated that the interaction between recombinant soluble VIP36 and BiP was divalent cation-dependent, and was little affected by ATP. The mode of the interaction is different from that observed between BiP and chaperone substrates, indicating that the interaction is constitutive. These results suggest that the complex of VIP36 and BiP may have an important role in protein quality control of secretory proteins in the cells.

P106
IMMUNOHISTOCHEMICAL STUDY OF THE BINDING OF THE ENZYMES EC-SOD AND PSEUDOEC-SOD TO HEPARAN SULPHATE ON ENDOTHELIAL CELL SURFACES

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Many degenerative processes and diseases such as aging and inflammatory actions have been coupled to the action of oxygen radicals. One important protective actor are the superoxide dismutases (SODs), which catalyzes the dismutation of superoxide radicals. One major site of oxidant attack is the endothelial cell surfaces, which is protected by extracellular SOD (EC-SOD).
EC-SOD binds to heparan sulphate on the cell surfaces, and the 26 C-terminal amino acids have been shown to be responsible for this binding. The affinity for heparin-sulphate has been partly characterized by us. Nine of the 26 amino acids are positively charged, however, nonspecific electrostatic interaction only explains a part of the affinity. Detailed characterization of this binding is essential for the understanding of hEC-SOD-related diseases and for the development of therapeutics.
The evaluation of the potential therapeutic value of EC-SOD and been hampered by failure of large-scale production.. To overcome this problem a fusion protein, mimicing human EC-SOD, was constructed. The characteristics of this PseudoEC-SOD closely resemble those of human EC-SOD, and preliminary results indicate a protective effect.
Blood vessels have been shown to contain relatively high levels of EC-SOD activity, but the specific localisation of EC-SOD in tissues is not well elucidated. Immunohistochemical detection of the localisation of human native EC-SOD and PseudoEC-SOD to cell surfaces of the human umbilical cord showed that human EC-SOD is located predominantly at the endothelial cell layer of the umbilical vessels, and that PseudoEC-SOD binds to cell surfaces in a very similar way as native EC-SOD does.

P107
RESPONSES TO ER STRESS OF INTRACELLULAR CARGO RECEPTORS

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In mammalian cells, the accumulation of unfolded proteins in the ER triggers unfolded protein response (UPR). The UPR affects several genes that encode ER chaperones and cargo receptors that assumed to be important after cell recovery from ER stress. The amount of mRNA of these proteins was measured by real-time PCR induced by tunicamycin, which leads to an accumulation of misfolded proteins in the ER by inhibiting N-glycosylation. Both ERGIC-53, a cargo receptor for the transport of glycoproteins from the ER to the Golgi, and VIP36, that for retrograde transport from the Golgi to the ER, were upregulated together with chaperones. In contrast VIPL, which is homologue of VIP36, was not upregulated by tunicamycin treatment for 42 hours. Interestingly, VIPL was coprecipitated with ERGIC-53 from the lysate of myc-VIPL and flag-ERGIC-53 coexpressing CHO cells. Further, we observed intracellular localization of ERGIC-53, VIPL, and several chaperones under ER stress by confocal scanning microscope. Based on these results, VIPL might play a different role from those of other L-type lectins.

P108
MOLECULAR CLONING AND CHARACTERIZATION OF ZEBRAFISH CL-P1 GENE

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Collectins are a family of C-type lectins which have collagen-like sequences and carbohydrate recognition domains (CRD). We previously reported that a new membrane type collectin CL-P1 which has not only a lectin activity but also a function as a scavenger receptor. We demonstrated that CL-P1 is expressed in vascular endothelial cells but not in macrophages. Scavenger receptors such as SR-AI and LOX-1 were found in rodent as the lowest animal. We tried to isolate a CL-P1 cDNA of zebrafish in order to analyze its physiological functions *in vivo*. Zebrafish CL-P1 (zCL-P1) CRD produced in *E.coli* and zCL-P1 on transfected cells specifically recognized Galactose and GlcNAc. zCL-P1 on cells also bound to microbes such as *Saccharomyces cerevisiae* and *Streptococcus aureus.* Moreover, it bound to oxidized low density lipoprotein (OxLDL). The patterns of these bindings were similar to human's ones. The expression of zCL-P1 mRNA has been found since 13 hpf (hours post fertilization). Immunohistochemical analyses in zebrafish showed CL-P1 was expressed in vascular endothelial area. The preliminary knockdown study using morpholino antisense oligonucleotides of zCL-P1 showed the insufficient body formation and angiogenesis. Moreover, we could rescue the vascular defect induced by zCL-P1 morpholino oligonucleotide in combination with zCL-P1 mRNA. These results suggest that CL-P1 is essential for zebrafish development during embryogenesis.

P109
DETECTION OF OLIGOSACCHARIDE LIGANDS FOR HGF, KGF, RANTES AND HEPARIN COFACTOR II BY NEOGLYCOLIPID MICROARRAYS OF GLYCOSAMINOGLYCAN-DERIVED OLIGOSACCHARIDE FRAGMENTS.

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The neoglycolipid technology for generating lipid-linked oligosaccharide probes has many features that render it adaptable for carbohydrate ligand detection. Dermatan sulfate (DS) is known to play an important role because of the ability to bind growth factors as well as chemokines and to modulate their biological activities during inflammation and response to injury. We prepared various iduronic acid-rich fragments from DS by complete digestion with chondroitinase ACI, and investigated whether the DS-binding proteins, such as HGF, RANTES, KGF and HCII, can detect their oligosaccharide ligands in neoglycolipid microarray. First, a comparison of the intensity of binding signals obtained from chondroitin oligosaccharides with those of heparin oligosaccharides showed that our microarray system is feasible not only to single-out the oligosaccharide ligands, but also to detect the difference between an intrinsic interaction unrelated only to electrostatic interaction and non-specific electrostatic interaction. Second, HGF, KGF and HCII showed preferentially binding to iduronic acid-rich fragments of DS oligosaccharides that are greater than 8-mers in length. In contrast, RANTES binding seemed to depend only on the negative charges; their binding intensity towards the DS oligosaccharides was somewhat stronger than the binding of HGF, KGF and HCII. Third, the use of polyvinylpyrrolidone and ovalbumin in place of BSA as a blotting agent was useful in these glycosaminoglycan dependent reactions for minimizing background due to non-specific interactions.

P110
HSC70 AND HSP70 GLCNAC-DIRECTED LECTINIC ACTIVITIES ARE MODULATED BY EXTRACELLULAR GLUCOSE RATE

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Heat shock proteins (HSP) are essential for cellular protection against a plethora of injuries. Recently we have shown that Hsc70 and Hsp70 (respectively the constitutive and the induced nucleocytoplasmic forms of 70kDa-heat shock protein family) share lectinic properties toward *O*-GlcNAc residues. These lectinic activities were enhanced by stress and especially during glucose deprivation (when compared to a normal glucose condition). Since *O*-GlcNAc synthesis directly depends on the extracellular glucose concentration through the hexosamine pathway, we postulated that Hsc70 and Hsp70 could modulate their lectinic activities according to extracellular glucose availability. For that purpose we have tested these lectinic properties after culturing HepG2 cells with a large panel of glucose concentrations or with an inhibitor of glucose transport (cytochalasin B) or with an inhibitor of glucose utilization (2-deoxyglucose). Hsc70 and Hsp70 strongly bound GlcNAc-labelled beads when glucose concentration was low and when the inhibitors were added to the culture medium. We have also shown that both Hsc70 and Hsp70 were *O*-GlcNAc modified but that their own glycosylation did not influence their GlcNAc-binding property by a possible self-recognition of an *O*-GlcNAc residue with the lectinic site.
Our results suggest that 70 kDa-Heat shock proteins could protects damaged proteins and cell integrity via target proteins *O*-GlcNAc residues considering that numerous studies tend to show that this glycosylation could be a protective signal toward proteasomal degradation.

P111
REGULATION OF ACTIVITIES OF METALLOPROTEASES MEPRIN α AND β BY MANNAN-BINDING PROTEIN

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Mannan-binding protein (MBP) is a C-type serum lectin that is known to be a host defence factor involved in innate immunity, and recognizes mannose, fucose and *N*-acetylglucosamine residues. Although some exogenous MBP ligands have been reported, little is known about its endogenous ligands. In the present study, we found that endogenous MBP ligands are highly expressed in the brush border epithelial cells of kidney proximal tubules by immunohistochemistry, and both meprin α and β (meprins) have been identified as novel endogenous MBP ligands through affinity chromatography and mass spectrometry. Meprins are membrane-bound and secreted zinc metalloproteases, which are extensively glycosylated and highly expressed in kidney and small intestinal epithelial cells, leukocytes and certain cancer cells. Meprins are capable of cleaving growth factors, extracellular matrix proteins, and biologically active peptides. Deglycosylation experiments indicated that the MBP ligands on meprins are high-mannose- or complex-type *N*-glycans. The interaction of MBP with meprins resulted in significant decreases in the proteolytic activity and matrix-degrading ability of meprins. Our results suggest that *N*-linked oligosaccharides on meprins are associated with the optimal enzymatic activity and that MBP is an important regulator for modulation of the localized meprin proteolytic activity *via N*-glycan-binding. Because meprins are known to be some of the major matrix-degrading metalloproteases in the kidney and intestine, MBP may contribute as a potential therapeutic target to tumor progression by facilitating the migration, intravasation, and metastasis of carcinoma cells, and to acute renal failure and inflammatory bowel diseases.

P112
GALECTIN-4 RECOGNIZES A VARIETY OF SULFATED COMPOUNDS

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Galectin-4 is highly expressed in the epithelial cells of the alimentary tract. In order to determine the multiple biological functions of galectin-4, first of all, we investigated the carbohydrate binding specificities. We found that galectin-4 specifically recognizes not only 3'sulfo core-1 and Gal-3-*O*-sulfated glycosphingolipids, but also cholesterol-3-sulfate, dextran-sulfate, and fucoidan, suggesting that the sulfate residues in these compounds are indispensable for the high-affinity binding to galectin-4. Galectin-4 consists of two tandem-repeated carbohydrate recognition domains. Our previous study indicated that GST-fused *C*-terminal domain showed several hundred times stronger affinity to 3'sulfo core-1 than GST-fused-*N*-terminal domain. Specific amino acids in the S3 β-sheet in the *C*-terminal domain are likely important for high affinity binding, since Gal-3-*O*-linked non-reducing terminal moieties such as sulfate or sialic acid seemed to interact with amino acids in the clefts formed by the S3 β-sheets. To determine the binding site for sulfated components, we prepared a site-directed mutant of GST-fused *C*-terminal domain of galectin-4, in comparison with amino acids on the S3 sheets of various galectins, and examined the binding ability to sulfated components. When the Lys residue in the S3 β-sheet was converted to Val, the mutant diminished the binding ability to ligands, indicating that the specific Lys residue is indispensable for recognition. The further examination by X-ray crystallographic analysis is proceeding.

P113
CRYSTAL STRUCTURE OF PSATHYRELLA VELUTINA LECTIN

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The lectin from the mushroom *Psathyrella velutina* recognizes specifically N-acetyl-glucosamine and sialic acid containing glycans. It has also been reported to bind pectin and heparin in a second independent site.
PVL has been purified from fungal fruiting bodies using affinity chromatography. In parallel, the gene has been sequenced showing high degree of polymorphisms. Crystallographic studies were performed on the lectin in the native state as well as on complex with GlcNAc and methyl-seleno-GlcNAc that allowed to phase the structure.
The crystal structure of the lectin is a single polypeptide of 401 residues arranged with a very regular 7-bladed *β*-propeller fold. The N-term region is located in the central cavity containing the pseudo seven-fold axis. In the complex, six GlcNAc residues are bound in pockets located between two

consecutive propeller blades. The β-hairpin loops connecting the two inner β-strands of each blade act as metal binding sites and two calcium ions were located in the structure. The multispecificity of this mushroom lectin combined with its similarity to one important class of cell adhesion molecules, integrins, is another example of the outstanding success of β-propellers as molecular binding machines in nature.

P114
THERMODYNAMIC AND STRUCTURES OF PSEUDOMONAS AERUGINOSA LECTINS AND THEIR INTERACTION WITH COMPLEX HUMAN GLYCOCONJUGATES

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Carbohydrate-mediated recognition plays an important role in the ability of pathogenic bacteria to adhere to the surface of the host cell in the first step of their invasion and infectivity. Together with carbohydrate receptors located on pili and flagellum, *Pseudomonas aeruginosa* also expresses soluble galactose- and fucose-binding lectins (PA-IL and PA-IIL). Complexes between both lectins and their monosaccharide ligands have been characterized by a variety of methods, including enzyme-amplification (ELLA), titration microcalorimetry, x-ray crystallography and molecular modeling. PA-IIL displays an affinity for fucose in micromolar range, unusually high for monosaccharide binding. These characteristics are correlated to the remarkable presence of two calcium ions in the binding site of the protein. When searching for ligands of biological interest, PA-IIL lectin displays highest affinity for lewis a, a trisaccharide located on epithelial cell glycoconjugates of CF patients, and human milk oligosaccharides. Combination of x-ray crystallography and titration microcalorimetry allow establish how fucosylated oligosaccharides can efficiently block the lectin, therefore providing the first structural clues for the role of human milk oligosaccharides in prevention of microbial infection. Based on these structural data, a large number of „glycomimetics“ compounds derived from fucose scaffold have been synthesized and tested for their affinity to PA-IIL. The best competitors are assayed for their potential use as anti-adhesion compounds.

P115
ADPATIN MEDIUM CHAIN 2 IS INVOLVED IN SCAVENGER RECEPTOR CL-P1-MEDIATED PHAGOCYTOSIS

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We previously reported a new type of scavenger receptor that human CL-P1, having a collectin structure. It could lead to phagocytose not only bacteria but also yeast. It bound oxidized low density lipoprotein (oxLDL).

In this study, we found that the μ2 subunit of adaptin complex was interacted with the intracellular domain of CL-P1 and its tyrosine motif recognition domain was directly associated with the intracellular domain of CL-P1 by using yeast two-hybrid and GST pull-down analyses. CL-P1-mediated yeast phagocytosis was inhibited by cytochalasin D and wortmannin same as Fcγ receptor-mediated phagocytosis. The binding of yeast was not increased by additional transfection of μ2 subunit in CHO/CL-P1 stable transfectants although that was increased by additional transfection of CL-P1 in these cells. It is surprising that the uptake of yeast was increased by additional transfection of μ2 as well as CL-P1. The uptake of yeast was not inhibited in any single amino acid substitution mutants in tyrosine sorting motif of CL-P1 but inhibited in truncated N-terminal 20 amino acids mutant of cytoplasmic domain of CL-P1.

Here we detected the μ2 subunit of AP-2 by yeast-two hybrid assay and demonstrated the interaction between CL-P1 and μ2 during the internalization of yeast. These results imply that AP-2 μ2 subunit might be involved in CL-P1 receptor-mediated phagocytosis and the N-terminal 20 amino acids cluster in CL-P1 was important for its phagocytosis.

P116
FLUORESCENCE POLARIZATION AS AN ANALYTICAL TOOL TO EVALUATE GALECTIN-LIGAND INTERACTIONS.

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Galectins are a family of β-galactose binding lectins associated with functions such as immunological and malignant events. To study the binding affinity of galectins for natural and artificial saccharides and glycoconjugates we have developed an assay using Fluorescence Polarization (FP). A collection of over 50 fluorescein conjugated saccharides were synthesized and used as probes with galectins-1, -2 -3, and -7, and each carbohydrate recognition domain of galectins-4, -8, and -9. Direct binding of a fixed probe amount with different amount of each galectin defined specificity and selectivity, and permitted selection of the optimal probe for inhibition

studies. Then a fixed amount of galectin and selected probe was used to screen the inhibitory potency of a library of non-fluorescent compounds. As the assay is in solution and does not require separation of free and bound probe, it is simple and rapid and can easily be applied to different unlabeled galectins. As all interacting components are known K_d values for galectin-inhibitor interaction can be directly calculated without approximation other than assuming a simple one site competition. The highest polarization approached (A_{max}) also may give information on the mode of binding.

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P117
MOBILITY MOMENT ANALYSIS OF LECTIN-SUGAR INTERACTIONS BY CAPILLARY ELECTROPHORESIS

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We always need to develop powerful and versatile research tools for protein-sugar interactions, which are still difficult to analyzeWe necessarily meet a variety of difficulties in the course of such pursuit because protein-sugar intereactions have inherent problems. Since endogenous counterparts for suga-binding proteins are usually heterogenous and obtainable only in very small quantity, we have to develop method

P118
STRUCTURES OF MANNAN-BINDING PROTEIN LIGAND OLIGOSACCHARIDES EXPRESSED ON SW1116 CELLS

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[Objective] Mannan-binding protein (MBP) is a C-type mammalian lectin specific for Man, GlcNAc, and Fuc. The serum MBP activates complement through the lectin pathway and is an important component associated with innate immunity. We have found that MBP has a potent growth inhibitory activity to a human colorectal carcinoma cell line *in vivo* via a complement-independent mechanism. We isolated and characterized the MBP-ligand oligosaccharides expressed on the surface of SW 1116 cells, which were assumed to be associated with the anti-tumor activity of MBP.
[Methods] The MBP-ligands on the surface of SW1116 cells were characterized with flow cytometry using plant lectins and anti-Lewis antibodies as inhibitors of FITC-MBP-binding to SW1116 cells. Pronase glycopeptides were prepared from whole cell lysates and oligosaccharides were liberated by hydrazinolysis followed by being tagged by pyridylamination. PA-MBP-ligand oligosaccharides were isolated with an MBP-affinity column, and then their sequences were determined by MS and MS/MS after permethylation, in combination with endo-β-galactosidase digestion, chemical defucosylation and lectin-HPLC analysis.
[Results and Discussion] The MBP-ligands were shown to be large, multi-antennary *N*-glycans carrying a highly fucosylated polylactosamine-type structure. At the non-reducing termini, Le^b-Le^a or tandem repeats of the Le^a structure prevail, a substantial proportion of which are attached via internal Le^x or *N*-acetyllactosamine units to the trimannosyl fucosylated core. It is concluded that MBP requires clusters of tandem repeats of the Le^b/Le^a epitope for recognition.

P119
MCFD2 ENHANCES SUGAR-BINDING ACTIVITY OF ERGIC-53

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ERGIC-53 is a mannose selective lectin with homology to plant leguminous lectins and considered to be transport receptor of several glycoproteins from the ER to the Golgi in mammalian cells. Recent studies on patients with the coagulation deficiency have indicated that MCFD2 might serve as a cooperator of ERGIC-53, but the relation between ERGIC-53 and MCFD2 is not fully understood. In this study, we investigated the influence of MCFD2 on carbohydrate-

binding activity of ERGIC-53. We prepared recombinant soluble ERGIC-53 protein (sERGIC-53) and MCFD2 protein expressed in *E.coli*. From the flow cytometric analysis using fluorescent-labeled sERGIC-53 and MCFD2, it was found that sERGIC-53 bound to the cell surface of HeLaS3 cells in the presence of MCFD2 but not in the absence of MCFD2. Ca^{2+} is necessary for the binding since EDTA destroyed the binding even in the presence of MCFD2. Inhibition experiment using glycopeptides from several glycoproteins revealed that the binding of sERGIC-53 to the cell surface in the presence of MCFD2 was mediated by high-mannose glycan. Treatment of the cells with deoxymannojirimycin, which inhibits the trimming of high-mannose glycan, increased the binding about three times. These results indicated that high-mannose glycan-binding activity of ERGIC-53 was enhanced by the engagement with MCFD2 in the presence of Ca^{2+}.

P119b
THE ROLE OF DISULFIDE BRIDGES AND GLYCANS FOR MURINE SIGLEC-4 FOLDING AND BINDING ACTIVITY

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A characteristic feature of all siglecs is the unusual distribution of cysteine residues in the two N-terminal domains leading to an intrasheet disulfide bridge in domain 1 and an unusual interdomain disulfide bridge between domains 1 and 2. To investigate the roles of these cysteine residues and the glycosylation in Siglec-4, we have generated mutant proteins of Fc-chimeras containing the N-terminal three domains replacing the cysteine residues by alanine or serine and deleting the *N*-glycosylation sites in any possible combination. These mutants were then analysed for expression in fibroblasts and binding activity.
None of the cysteine mutants was secreted in significant amounts. Pulse-chase experiments demonstrated that the proteins were degraded within the cells, suggesting that the interdomain disulfide is necessary for folding. Whereas binding of the wildtype protein was almost unchanged in the presence of dithiothreitol or glutathione as determined by STD-NMR, low concentrations of mercaptoethanol lead to a loss of activity. Most likely, for an active conformation only the intradomain disulfides are essential, since these are probably only accessible for mercaptoethanol. Also changes in glycosylation had an effect on expression levels, although to a much milder extent. Apparently, mainly the single glycan in domain 1 is necessary for effective folding. Processing to complex glycans has no effect, since fibroblasts with a processing defect expressed protein at high levels similar to wildtype cells. Finally, none of the glycans is needed to maintain an active conformation, since enzymatic removal of all glycans from the protein produced did not lead to a significant reduction in binding.

P120
LACTAMIZED SIALYL 6-O-SULFO PARAGLOBOSIDE & LEWIS X NEOGLYCOLIPIDS: SYNTHESIS AND ANTIGENIC REACTIVITY AGAINST G159 MONOCLONAL ANTIBODY

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It has been demonstrated that sialyl 6-O-sulfo Lewis X is an endogenous L-selectin ligand on the human high endothelial venule[1]. De-N-acetyl-sialyl 6-O-sulfo Lewis X was also found to be a superior ligand for L-selectin, which may be inactivated (down regulation) by conversion into the cyclic (lactam) structure being detected with G159 monoclonal antibody[2]. To investigate the minimal structure required for recognition by G159 mAb, we have synthesized the lactamized sialyl 6-O-sulfo Lewis X (tetrasaccharide) B-30 derivative[3]. Contrary to our expectation, it was not recognized by G159 mAb to suggest the importance of the lactose moiety for the recognition. The synthesis of the title compounds was achieved by the coupling of NeuTFAc-α(1,3)-Gal imidate with the suitably protected GlcNAc-β(1,3)-Gal-β(1,4)-GlcOSE derivative. Both lactamized sialyl 6-O-sulfo paragloboside and Lewis X (hexasaccharide) neoglycolipids were clearly recognized with G159 mAb showing that both the cyclic neuraminic acid and the sulfate group at O-6 of GlcNAc would be involved in the G159-defined determinant.

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P121
NOVEL CARBOHYDRATE STRUCTURES RECOGNIZED BY A HUMAN-SPECIFIC MONOCLONAL ANTIBODY 1CF11

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A human-specific monoclonal antibody 1CF11, which was originally obtained as a mAb against human milk MUC1 mucin, recognizes a common carbohydrate epitope shared by various glycoproteins in human secretion[1]. To determine the epitope structure, we have attempted to isolate the fragment bearing 1CF11 epitope from human milk lactoferrin (hLf), which was first digested by pronase and applied to a Superdex 75 column. The fragments carrying 1CF11 epitope were isolated by using Con A-Sepharose column, followed by affinity purification on 1CF11 antibody column, to give two glycosylated peptides corresponding to SSQE (Pep1) and APGS (Pep2). These glycosylated peptides were each subjected to MALDI-TOF MS analysis, hydrazinolysis and p-aminobenzoic acid ethyl ester (ABEE) labeling analysis. As a result, the existence of two glycans corresponding to Glc-Glc- and Man-Glc-disaccharides has been suggested. In the inhibition ELISA assay using oligosaccharide libraries, Glcα(1,3)Glc (nigerose) and Glcα(1,3)Glcβ- structure was found to be the mot potent inhibitor against 1CF11 mAb. This result suggests that the glucose-linked novel human-specific O-glycans (a glucose-linked fundamentally unveiled disaccharide; GIFU) may widely be occurred in human secretion.

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P122
THE MEMBRANE TYPE COLLECTIN CL-P1 IS UP-REGULATED IN ISCHEMIA/REPERFUSION CONDITION

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[Introduction] CL-P1 is a new scavenger receptor which is a membrane type collectin from placenta. We examined the influence of ischemia/reperfusion on expression of CL-P1 in both *in vitro* and *in vivo* and investigated the relation between CL-P1 and atherosclerosis. [Experimental approaches] On the *in vitro* model, the HUVECs were cultured in the environment and reoxygenation condition. On the *in vivo* model, the right common carotid artery of SD rat was occluded with two clips (ischemia), removed (reperfusion), and extirpated after several periods. For the evaluation of treated vessels, we injected DI labeled oxidized LDL into the vessels and evaluated them by microscopic analyses. [Results]Hypoxia/reoxygenation stress significantly increased CL-P1 mRNA expression in HUVECs, maximum at 72 hs and it was observed continuously at 120 hs after reoxygenation. On the ischemia/reperfusion model, rat CL-P1 mRNA was elevated and peaked at 3 days. Immunohistochemical analyses revealed that CL-P1 was remarkably expressed in the intima, peaked at 7 days and remained on 14 days after reperfusion. Oxidized LDL was remarkably caught in the intima of the ischemia/reperfusion vessels.[Conclusions]Our results demonstrate the ischemia/reperfusion can induce CL-P1 mRNA and protein in both *in vitro* and *in vivo* conditions. In addition, the uptake of oxidized LDL, which plays crucial roles in the pathogenesis of atherosclerosis, with intima seems to be correlated with the induced CL-P1. We propose that these observations may have important implications with regard to endothelial dysfunction and even atherosclerosis.

P123
SYNTHETIC N-GLYCANS FOR QUANTITATIVE ANALYSIS OF CALRETICULIN

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Among various functions of glycoprotein glycan chains, the roles of asparagine (N-) linked oligosaccharides in glycoprotein quality control system are attracting particular attention. In order to gain precise understanding of the system, homogeneous and structurally defined oligosaccharides are highly desired. Recently, we established a highly stereoselective synthetic route for the series of ER related N-linked oligosaccharides (1-3). These oligosaccharides will be useful as molecular probes to clarify various issues related to glycoprotein quality control sysem, including protein-oligosaccharide interactions and substrate specificity of processing enzymes.
In this presentation, we wish to report the following two topics; 1) the systematic syntheses of ER related high mannose type glycan chains ($Glc_{0\sim3}Man_{7\sim9}GlcNAc_2$) using convergent manner and 2) interaction analysis of synthetic oligosaccharides with calreticulin (CRT), the lectin like chaperone, using isothermal titration calorimetry. The interaction studies revealed that CRT binds mono-glucosylated oligosaccharides. However, the binding constants varied strongly depending on the sugar chain structure of non-reducing end (B or C arm). Based on these results, the structure requirement for the recognition of CRT will be discussed.

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P124
GANGLIOSIDE BINDING SITES IN INTERLEUKIN-4 MOLECULE: A PHOTOAFFINITY STUDY

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Gangliosides have a multitude of functions in maintaining immune homeostasis but they are known also as immunosuppressors; the main mechanism of the inhibition includes direct ganglioside/interleukin interaction with formation of inactive complex. Earlier, we have shown that gangliosides bind to interleukin-2 and interleukin-4 (IL-4) with high constants close to that for the cytokines' binding with their receptors. We have studied the ganglioside GM1 binding to IL-4 by photoaffinity labeling using newly prepared probe, ganglioside analogue ^{125}I-Dcp-GM1, bearing photoaffine Dcp group in the polar head of the molecule.
After incubation of the probe with IL-4 and subsequent photolysis, the cross-linking products, after partial proteolysis, were analyzed by HPLC and MALDI-TOF MS. Cross-linked peptides have masses increased against initial Dcp-derived (Dcp minus N_2) group or the group plus neuraminyl residue. We suggest that molecular peak can be presented in mass spectra as $[M + H]^+$, $[M + Na]^+$ or $[M + K]^+$ species. Search of the heavier fragments was unsuccessful. The mass spectrometry analysis of modified peptides shows three interleukin fragments, QKTLCTE, KE, and ANQSTLENFLE; all of them belong to loops of cytokine molecule with high structural mobility.
To interpret these data, the known model of IL-4 molecule was used. Graphic simulation shows that IL-4 has two ganglioside binding sites at the opposite regions of the molecule. One site constrains enter to the hydrophobic cavity of IL-4, and another constrains enter to the same hydrophobic cavity from the back side.
Support: ISTC #2185.

P125
THE SEARCH FOR THE TUMOR CELL LECTINS WITH THE HELP OF PHOTOAFFINITY NEOGLYCOLIPID PROBES

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Earlier, we equipped liposomes loaded with anticancer drugs with specific carbohydrate residues (vectors) as targeting ligands for lectins overexpressed on tumor cells. The assay of carbohydrate specificity of cultured cells was performed with fluorescent carbohydrate probes.
To study the mechanism of vectored liposome/cell interaction, and "catch on" cell membrane receptor(s), we have carried out a series of experiments *in vitro* on photoaffinity labeling (PAL) of melanoma M3 cells by liposomes bearing neoglycolipid probes with determinants trisaccharide A (**I**, Fucα1-2(GalNAcα1-3)Galβ) and Sialyl Lewis X (**II**, Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ), and promyeloid HL-60 cells – by liposomes with Sulphate Lewis A (**III**, 3'HSO_3-Galβ1-3(Fucα1-4)GlcNAcβ). The probes are conjugates of glycosides and *rac*-1,2-dioleoylglycerol connected *via* oligoethyleneglycol spacer, and have close to carbohydrate moiety the carbene-generating ^{125}I-diazocyclopentadien-2-ylcarbonyl group. To detect binding just on the cell surface, short incubation times (5–10 min) in the presence of increasing free carbohydrate concentrations were used. After photolysis, PAGE autoradiography of cell lysates showed radiolabeling of a number of protein bands was detected, the profiles differing substantially for different probes and cells. In spite of careful delipidation before SDS-PAGE, the radiolabeling patterns were complicated by the background of free glycolipid probes or their cross-links. The non-specific binding was assessed by PAL with inactive pentaol-equipped liposomes. Detailed analysis of the phoregrams revealed the probe-binding proteins: on the M3 cells with probe (**I**) – minor protein of ~18 kD; with probe (**II**) – proteins of ~43, ~60 (maximal), ~64 and ~80 kD; on the HL-60 cells with probe (**III**) – proteins of ~36, ~43, ~48, ~54, ~66 kD, and in zone 75–85 kD. Comparison of our and literature data let us to suppose that detected receptors mediating the interaction with the vectored liposomes may be attributed to some known lectins of mammalian cells.

P126
STRUCTURAL ANALYSIS OF *N*-LINKED OLIGOSACCHARIDES IN HUMAN PLACENTA AND UMBILICAL CORD

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We have comparatively analyzed *N*-linked oligosaccharides expressed in human placenta and umbilical cord. Oligosaccharides were liberated from lyophilized placenta and umbilical cord by hydrazinolysis. After acetylation of free amino groups, the released *N*-linked oligosaccharides were pyridylaminated, and the resultant pyridylaminated (PA) oligosaccharides were analyzed by size-fractionation HPLC. As a result, one characteristic *N*-linked oligosaccharide was found to be unique to placenta and two were so to umbilical cord. Thus, these oligosaccharides specific for placenta and umbilical cord were further purified by reversed phase, size-fractionation, and anion exchange HPLC. Structures of *N*-linked oligosaccharides were determined by 2-demensional sugar mapping, sequential exoglycosidase digestions,

MALDI-TOF mass, and analytical lectin-affinity chromatography (FAC). The chemical structure of *N*-linked oligosaccharide characteristically expressed in human placenta was mono sialylated (alpha2-6) biantennary complex-type oligosaccharide, and those specific for umbilical cord were mono sialylated (alpha2-3 or 2-6) fucosylbiantennary complex-type oligosaccharide. Linkages of sialic acids were determined by using linkage-specific sialidases, and were further confirmed by FAC with alpha2-3 specific lectin (galectin-8, N-CRD) and alpha2-6 specific lectin (SSA). Biological significance of the presence of these monosialylated complex-type *N*-linked oligosaccharides will be discussed.

P127
MOLECULAR CHARACTERIZATION OF FUNCTIONAL DOMAINS OF MEMBRANE TYPE COLLECTIN CL-P1

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Collectins are a family of proteins that have at least two characteristic structures, a collagen-like domain and a carbohydrate recognition domain (CRD). They are involved in host defense since they can bind to carbohydrate antigens. Recently we found a new membrane type collectin CL-P1 similar to scavenger receptor, SR-AI. Expression studies using CHO cells showed that CL-P1 can bind and phagocytose bacteria and yeast as well as oxidized low density lipoprotein (OxLDL). CL-P1 is a multifunctional molecule having activities of lectin and scavenger receptor. In this study, we investigated the functional domain of CL-P1 by using soluble CL-P1s having the extracellular domain and the membrane CL-P1s. Both soluble type and membrane binding type CL-P1 indicated same binding activity of sugar. We made deletion mutants of functional domain, collagen-like domain and/or CRD. CL-P1 lacking collagen-like domain could not bind to OxLDL and microbes. The cluster of positive charged amino acids in collagen-like domain was known to important for binding to ligands as scavenger receptors, SR-AI and MARCO. Furthermore we made single amino acid substitution mutants in positive charged amino acids and investigated which cluster was important to bind to ligands. Here we would like to demonstrate a functional domain and key cluster of positive charged places in CL-P1.

P128
PURIFICATION OF A NEW MARINE LECTIN FROM JAPANESE SPONGE *HOLICHONDRIA OKADAI.*

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A lectin are isolated from the Japanese sponge *Halichondria okadai* by the combination using lactose-conjugated affinity chromatography, ion-exchange chromatography, and gel permeation chromatography. Corrected sponge was homogenized with 150mM NaCl containing 20mM Tris-HCl (pH 7.4) (TBS) in motor and the supernatant was obtained by the centrifugation. The crude extract was applied to lactose-conjugated column and the column bound protein was eluted with 50mM lactose containing TBS. The purified lectin was active even in the absence of divalent cations nor reducing reagent and presented a single 30kDa band by SDS-PAGE both reducing and non-reducing conditions. The gel-permeation chromatography by FPLC system connected with Superdex G-75 column appeared the protein 60,000 Da both in the presence and absence of haptenic sugar in the running buffer by the using five standard proteins at the range from 66,000 to 14,000. The result suggested that the lectin was a non-covalently bound dimmeric molecule consisted of 30kDa polypeptide. The lectin appeared to recognize not only beta galactoside but also alpha galactoside by hemagglutinating inhibition assay. We analyzed that the binding specificity of four galactose binding lectins including the sponge lectin with some glycoproteins using SPR-biosensor, presenting that the lectin bound well with lacto-N-fuco pentaose as difference from RCA120.

P129
TRITYROSYL MOTIF OF ENAMEL PROTEIN AMELOGENIN THAT BINDS TO GLCNAC > NEUAC AND GLCNAC-MIMICKING PEPTIDES SPECIFICALLY RECOGNIZES SULFATED-GLCNAC.

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The major enamel protein amelogenin possesses lectin-like properties, which is attributed to the presence of a minimal peptide sequence called the amelogenin tyrosyl motif peptide (ATMP) with affinity for N-acetyl glucosamine (GlcNAc) > N-acetyl neuraminic acid (NeuAc)[1]. This motif is capable of recognizing the GlcNAc residues of glycoconjugates, GlcNAc-mimicking-peptides and their carrier-proteins found in ameloblasts and in enamel matrix[2-5]. Therefore, identification of the nature of interactions of amelogenins with structural analogs of GlcNAc and NeuAc and their glycosidic linkages found in enamel or dentin and cell surface glycoconjugates in the developing tooth is critical for an understanding of normal and pathological enamel formation. We have recently observed the presence of sulfated-NeuAc/GlcNAc associated with Keratan sulfate (KS) in dentin

and the dentin-enamel junction but not in predentin[6]. We hypothesize that ATMP may specifically recognize the sulfated GlcNAc/NeuAc during enamel formation. We have tested the hypothesis by examining the affinity of recombinant amelogenin, rM179 to GlcNAc carrying phosphate or sulfate at position 1 or 3 or 6. Presence of sulfate at position 3 but not at position 1 or 6 or phosphate residues of GlcNAc abrogated the binding affinity. These observations suggest that amelogenin, a multifunctional protein may interact with carbohydrates/proteins through these ligands that may direct the downstream events of enamel mineralization during amelogenesis in a cooperative manner. [1]*J. Biol. Chem.* 274: 2464, 1999;[2]*J. Biol. Chem.* 275:39654, 2000;[3]*J. Biol. Chem.* 276:36586, 2001;[4]*J. Biol. Chem.* 278: 20293, 2003;[5]Biochem Biophys Res Commun. 323:1075, 2004;[6]Acta Histochem. 107:43, 2005. Supported by NIH-NIDCR DE-13204.

P130

BINDING SPECIFICITY AND SITE-DIRECTED MUTAGENESIS OF THE N-TERMINAL DOMAIN OF RECOMBINANT HUMAN GALECTIN-4 SUGGEST TWO ALTERNATIVE CORE GALACTOSE BINDING MODES.

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Galectin-4 is a bi-CRD galectin found in the gastrointestinal tract, where it has been reported to be a cell membrane raft stabilizer and to take part in cancer and immunity. We have studied the binding specificity of the N-terminal domain of galectin-4 using fluorescence polarisation and related the results to a model with five binding subsites (A-E), where site C is the conserved defining characteristic for galectins that binds galactose. Galectin-4N showed significantly lower affinity for lactose and LacNAc (in site C-D) compared to many other galectins, e.g. galectin-3. The highest affinity was for blood group A and H like determinants, with extensions into site B. One of the key amino acids in site B, Phe146, has an unusually large side chain compared to other galectins. When this was substituted to an Ala as found in the corresponding position in galectin-3, affinity for lactose and compounds with lactose in site C-D increased. Surprisingly, the affinity for compounds with LacNAc in site C-D did not increase. To explain how a change in site B can thus affect interactions in site D (with the Glc or GlcNAc of lactose or LacNAc, respectively) we suggest a model of two alternative ways of binding galactose in site C in the CRD. Finding the ligand binding properties of galectin-4 may help our understanding of its crucial role as a cell membrane raft stabilizer, and provide the information needed to design the optimal blocking substances such as anti-tumoral drugs.

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P131

PRESENCE AND AMOUNTS OF STARCH GRANULE SURFACE PROTEINS IN VARIOUS STARCHES

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Presence of starch granule surface proteins was ascertained in wheat, barley, maize, rice, prosomillet, chestnut, acorn, tapioca and potato starch by staining with fluorescent dye, fluoroscamine and observation under a fluorescence microscope. The amount (%) of these starch granule surface proteins was determined by a dye-binding method, and compared with the amount (%) of total proteins (N x 5.7). These findings suggested that almost all of the starch proteins may be present on the starch granule surface.

P132

CARBOHYDRATE RECOGNITION FACTORS OF THE LECTIN DOMAIN IN RICIN: A TOXIC PROTEIN FROM THE SEEDS OF *RICINUS COMMUNIS*

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Ricin is a potent cytotoxic protein with lectin domains produced in the seeds of the castor bean *Ricinus communis*. To corroborate the biological properties of ricin, it is essential to understand the recognition factors involved in the ricin-glycotope interaction. In previous studies, the knowledge of the binding properties of ricin was limited to oligosugars with different specificities. Here, the recognition factors of lectin domains in ricin were examined by enzyme-linked lectinosorbent (ELLSA) and inhibition assays, according to the mammalian Gal/GalNAc structural units and corresponding polyvalent forms. Except for blood group GalNAcα1-3Gal (**A**) active and Forssman (GalNAcα1-3GalNAc, **F**) disaccharides, ricin has a broad range of affinity for mammalian disaccharide structural units — Galβ1-4Glc

(**L**), Galβ1-4GlcNAc (**II**), Galβ1-3GlcNAc (**I**), Galβ1-3GalNAcα1- ($\mathbf{T}_{\alpha}$), Galβ1-3GalNAcβ1- ($\mathbf{T}_{\beta}$), Galα1-3Gal (**B**), Galα1-4Gal (**E**), GalNAcβ1-3Gal (**P**), GalNAcα1-Ser/Thr (**Tn**) and GalNAcβ1-4Gal (**S**). Among the polyvalent glycotopes tested, it reacted best with type **II** several **T**, **Tn** and blood group Sd. (a^+)-containing glycoproteins. Except for bird nest and Tamm-Horsfall gps (THGP), this lectin reacted weakly or not at all with **ABH**-blood type and sialylated gps. From the present and previous results obtained, it can be concluded that: (i) the combining sites of these lectin domains should be a shallow-groove type, recognizing Galβ1-4Glc as the major binding site; (ii) its size may be as large as a tetrasaccharide and most complementary to lacto-*N*-tetraose (Galβ1-3GlcNAcβ1-3Galβ1-4Glc) and lacto-*N*-neotetraose (Galβ1-4GlcNAc β1-3Galβ1-4Glc); (iii) the polyvalency of glycotopes, in general, enhance the binding; (iv) a hydrophobic interaction in the vicinity of the binding site for sugar accommodation increases the affinity for Galβ-.

P133
EFFECT OF OLIGOMERIZATION AND GLYCOSYLATION ON SOYBEAN AGGLUTININ (SBA) STABILITY.

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Soybean agglutinin is a tetrameric legume lectin each of whose subunits are glycosylated. This protein shows much higher stability when compared to the proteins of the same family due to the high degree of subunit interactions across the non-cannonical interface. Further, the glycosylation in this protein is seen at the interface (at Asn-75) and the glycan of one subunit makes substantial contacts with the amino acid residues of the other subunit. The stability thermodynamic parameters of the monomeric of SBA when compared to the tetrameric form (both obtained from solution denaturation studies) shows that the stability of the tetramer is much more than four times the stability of the monomer. This gives an estimate of the degree of stability that inter-subunit interactions have imparted to the system. The monomeric species is found at pH 1.9 as evident from size exclusion chromatographic studies and dynamic light scattering studies. The analyses of CD and fluorescence spectroscopy suggest that the monomer is well folded and it has a very different characteristic feature when compared to the tetramer. The T_g difference in the two forms of the protein is ~40K, while the difference in ΔC_p is only 1.6kcal/mol/K. this suggests that the major hydrophobic core is present in the monomer and oligomerisation involves mainly ionic interactions.

Acknowledgements: DBT (Department of Biotechnology), Government of India

P134
EFFECT OF *N*-LINKED GLYCOSYLATION ON ENDOTHELIAL LIPASE-MEDIATED REMODELLING OF APOLIPOPROTEIN E CONTAINING RECONSTITUTED HIGH DENSITY LIPOPROTEIN

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Apolipoprotein E (apoE) plays a critical role in high density lipoprotein (HDL) metabolism.[1] It exists in three different isoforms, apoE2, apoE3, and apoE4, and is primarily transported in plasma as a component of HDL. HDL subpopulations that contain apoE do not contain either apoA-I or apoA-II. Endothelial lipase (EL) is a member of the triglyceride lipase gene family that has high phospholipase and low triglyceride lipase activity. It is a 68kDa glycoprotein bearing four N-glycans, which are known to effect both the secretion and lipolytic activity of EL.[2]

We have shown previously that apolipoproteins regulate the kinetics of both EL- and HL-mediated remodelling of reconstituted HDL (rHDL), [3,4] and that the presence of *N*-glycans on EL alters both enzyme activity and substrate specificity for rHDL containing apoA-I and/or apoA-II. Herein we describe how *N*-linked glycosylation effects EL-mediated phospholipid hydrolysis in apoE-containing rHDL.

A eukaryotic expression system was used to determine the effect of each glycosylation site on the phospholipase activity of EL. Site-directed mutagenesis of asparagine (N) to alanine (A) gave four mutants each lacking one glycan, EL(N62A), EL(N118A), EL(N375A), and EL(N473A). These mutants were used to determine the kinetics of phospholipid hydrolysis in (E2)rHDL, (E3)rHDL, and (E4)rHDL.[4] Hydrolysis was assessed as the amount of non-esterified fatty acid (NEFA) liberated during incubation with a constant amount of EL. A comparison of mutant and wild-type EL-mediated phospholipid hydrolysis of the various rHDL types will be presented, along with details of the subsequent kinetic analysis, which revealed that removal of the carbohydrate moiety at each of the four *N*-linked glycosylation sites has varying effects on the activity and/or specificity of the enzyme.

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P135
OBSERVATION OF DOMAIN SWAPPING AS A MECHNISM FOR ENHANCING HEPARIN BINDING:

CASE STUDY OF THE HATH DOMAIN OF HUMAN HEPATOMA-DERIVED FROWTH FACTOR

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Hepatoma-derived growth factor (HDGF)-related proteins (HRPs) comprise a new growth factor family sharing a highly conserved N-terminal HATH domain. The exogenously treatment of HDGF is transported to cytosol through the binding effect of N-terminal HATH domain to both high affinity membrane receptor and high capacity heparan sulfate on cell surface. Here we further demonstrate that HATH domain can form a domain-swapping dimer under physiological conditions that the HATH-dimer can interact with heparin with two orders of magnitude (K_d ~ 13 nM) higher than that of the monomer (K_d ~ 1.2 μM). NMR intermolecular NOEs reveal that the structural elements of β-sheet 5, helix A and helix B are involved in domain swapping that loop 4 plays the role as a hinge loop. Chemical shift comparison between monomer and dimer shows that the loop 2 region undergoes the most significant structural perturbation, implying the region of dimer interface. The lengths of heparin oligomers bound to per HATH-monomer and HATH-dimer were estimated to be ~ 5 and ~ 10 saccharide units, respectively. Thus, the heparin-binding site of the dimer is roughly twice as long as that of the monomeric form. Based on the proposed structural model, this can be accomplished by attaching two HATH monomers and align the two binding sties in a serial manner. The two-order magnitude of heparin-binding enhancement can be accounted by the well-known multivalency effect. The discovery of domain swapping in HATH-dimer as a mechanism for enhancing heparin binding and for regulating the function of growth factor provide new leads for understanding the molecular mechanism of protein-carbohydrate interaction on cell surface.

P136
THERMODYNAMIC ANALYSIS OF CARBOHYDRATE AND PORPHYRIN BINDING TO *MOMORDICA CHARANTIA* (BITTER GOURD) LECTIN

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Binding of saccharides and porphyrins to the *Momordica charantia* lectin (MCL) was investigated by isothermal titration calorimetry (ITC) and difference absorption spectroscopy, respectively. ITC studies show that the tetrameric MCL binds two sugar molecules with binding constants (K_b) in the range of 7.3×10^3 to 1.52×10^4 M^{-1}. The binding reactions were enthalpy driven (ΔH_b = -50.99 to -43.39 $kJ.mol^{-1}$) and the entropy of binding was negative (ΔS_b = -99.2 to -72.0 $J.mol^{-1}.K^{-1}$). Changes in heat capacity for the binding of disaccharides were significantly larger than those obtained for the monosaccharides and could be correlated with ligand surface areas. Absorption changes in the *Soret* band region were monitored to characterize the binding of porphyrins to MCL. The tetrameric MCL binds four porphyrin molecules and the stoichiometry was unaffected by the presence of lactose, indicating that porphyrin and carbohydrate ligands bind at different sites. Both cationic and anionic porphyrins bind to the lectin with comparable affinity (K_a = 10^3 – 10^5 M^{-1}). The thermodynamic parameters associated with the interaction of several porphyrins indicate that porphyrin binding to MCL is enthalpically driven with negative contribution from binding entropy. Enthalpy-entropy compensation was observed in the interaction of both carbohydrates and porphyrins with MCL, underscoring the role of water structure in the overall binding process. Analysis of CD spectra of MCL indicate that this protein contains about 13% α-helix, 36% β-sheet, 21% β-turn and rest unordered structures. Binding of neither carbohydrates not porphyrins significantly altered the secondary and tertiary structures of MCL.

P137
CV-IIL: NEW LECTIN FROM HUMAN OPORTUNISTIC PATHOGEN CHROMOBACTERIUM VIOLACEUM

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Pathogen adhesion to the host cell surface followed by its colonisation, invasion and infectivity is usually mediated by protein-carbohydrate interactions. *Pseudomonas aeruginosa*, an opportunistic pathogen responsible for numerous nosocomial infections in immunocopromised patients, produces a variety of carbohydrate-binding proteins that could be involved in host recognition and adhesion. One of them, PA-IIL, is a fucose binding lectin that is closely related to the virulence of the bacterium [1] Searching in databases for proteins displaying sequence similarities to PA-IIL revealed new homologous proteins in other opportunistic pathogens like *Chromobacterium violaceum*. Human infection by *Ch. violaceum* is rare but when it occurs, it is associated with very high mortality rate [2]. The gene *cv1741* coding a hypothetical protein similar to PA-IIL, was cloned and the resulting

recombinant protein, CV-IIL, has been expressed in *E. coli* and purified to homogeneity. inding affinities of the protein with different mono and oligosaccharides using ELLA (enzyme linked lectin assay) and isothermal titration microcalorimetry have been characterized and crystal structures of CV-IIL in complexes with α-D-methylmannoside and α-L-methylfucoside have been solved. The binding data together with structure analysis allowed comparison of CV-IIL and PA-IIL and brought more detailed view on fine specificity of both lectins. Structure basis of the protein/sugar complexes and the thermodynamics of their interactions could be helpful to design carbohydrate-based compounds that can be used as alternatives to antibiotics or new antiadhesive therapeutics.

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P138
THE UNUSAL BINDING SPECIFICITY OF A FUCOLECTIN-RELATED PROTEIN OF UNKNOWN FUNCTION ENCODED BY THE SP2159 GENE OF STREPTOCOCCUS PNEUMONIAE

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The fucolectin-related protein of unknown function (FRP) encoded by the SP2159 gene of S. pneumoniae[1] shares 34% identity with clostridial blood group A- and B-cleaving endo-β-galactosidase[2]. To study the binding specificity of FRP, we have cloned the FRP gene and expressed the recombinant protein in E. coli. Using the biotin/avidin-mediated microtiter plate lectin-glycan binding assay in conjunction with the inhibition of agglutinin-glycan interaction, FRP was found to bind well with Le^y/Le^b [Fucα1,2-Galβ1,4/3(Fucα-1,3/4)GlcNAc], A_h [GalNAcα1,3(L-Fucα1,2) Gal]/Le^y , B_h [Galα1,3(L-Fucα1,2)Gal]/Le^y and H [L-Fucα1,2Gal] active glycoproteins (gps), but not with Le^a/Le^x [Galβ1,3/4(Fucα1,4/3)GlcNAc] gps, nor their precursors [Galβ1,3/4GlcNAc], or other mammalian glycotopes. Among the glycan ligands tested, Le^y/Le^b, H, A_h/Le^y and B_h/Le^y active gps were found to be potent inhibitors for FRP-glycan interaction, and the gluco-analogue of the Le^y hapten [Fucα1,2Galβ1,4(Fucα1,3)Glc] was the best inhibitor. This inhibitor was 2, 16 and 24 times more potent than Le^b hexasaccharide [Fucα1,2Galβ1,3(Fucα1,4)GlcNAcβ1,3Galβ1,4Glc], Fucα1,2Gal, and L-Fuc, respectively. In the case of monsaccharide inhibitors, L-Gal was about 4.8, 60 and 88 times better inhibitor than L-Fuc, D-Ara and D-Gal, respectively. From these results, we conclude that: (i) Le^y, Le^b and H determinants, containing the monomeric forms of Fucα1,2Gal-glycotope, were weak ligands for FRP, but their corresponding polyvalent forms exhibited strong and visible interactions with FRP; (ii) although Le^x [Galβ1,4(Fucα1,3)GlcNAc], Le^a [Galβ1,3(Fucα1,4)GlcNAc], A (Gal NAcα1,3Gal) and B (Galα1-3Gal) were poor inhibitors, they enhanced the FRP-Fucα1,2Gal glycotope binding.

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P139
RETARDATION OF REMOVAL OF RADIATION–INDUCED APOPTOTIC CELLS IN DEVELOPING NEURAL TUBES IN MACROPHAGE GALACTOSE-TYPE C-TYPE LECTIN-1 (MGL1/CD301A)-DEFICIENT MOUSE EMBRYOS

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Apoptotic cells in embryos are known to be removed by phagocytes. Recognition of glycosylation changes may represent one of the molecular mechanisms used for the phagocytosis. We hypothesized that apoptotic cells generated by X-ray irradiation in developing embryos are recognized by MGL/CD301, the macrophage galactose-type C-Type lectin. In mice, the presence of two isotypes, MGL1/CD301a and MGL2/CD301b is acknowledged. In the present study, embryos in pregnant mice with *Mgl1*+/- background that had been mated with *Mgl1*+/- or *Mgl1*-/- background males were used to assess a hypothesis that this molecule plays an important role in the clearance of apoptotic cells. After X-ray irradiation at 1 Gy of developing embryos at 10.5 d.p.c, the number of *Mgl1*-/- pups was significantly reduced as compared to *Mgl1*+/+ pups after X-ray irradiation. Distributions of MGL1- and MGL2-positive cells and apoptotic cells were histologically examined in irradiated

Mgl1+/+ embryos. MGL1-positive cells were detected in the neural tube, where cells undergo apoptosis, whereas MGL2-positive cells were not observed. Biotinylated recombinant MGL1 bound a significant portion of the apoptotic cells. When *Mgl1*+/+ and *Mgl1*-/- embryos were examined for the presence of apoptotic cells, the clearance of apoptotic cells was slower in *Mgl1*-/- embryos than in *Mgl1*+/+ embryos. These results strongly suggest that MGL1/CD301a is involved in the clearance of apoptotic cells, that is essential in the repair and normal development of X-ray irradiated embryos.

P140
ANTIBODY RECOGNITION OF CARBOHYDRATE AND PEPTIDE LIGANDS STUDIED BY MOLECULAR DOCKING: IMPLICATIONS FOR XENOTRANSPLANTATION

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Introduction. Natural or preformed antibodies that react with oligosaccharides bearing the terminal galactose-α(1,3)-galactose [Galα(1,3)Gal] epitope (major carbohydrate xenoantigen) are present in the sera of all humans (anti-Gal antibodies). These antibodies initiate hyperacute rejection of xenografts of porcine organs in human recipients. Management of the deleterious effects of this interaction represents the first major obstacle for successful engraftment. Because of many difficulties associated with the use of complex carbohydrates as therapeutics, the search for alternative agents had been carried out and resulted in the identification of peptides that inhibit the interaction of anti-Gal antibodies with Galα(1,3)Gal. *Experimental procedures.* Structural aspects of antibody recognition of carbohydrate xenoantigens and their peptide mimics have been studied by automated molecular docking. *Results.* Docked complexes of monoclonal anti-Gal antibodies with Galα(1,3)Gal, its GlcNAc derivative and the peptide mimic have been generated. Critical ligand-protein hydrogen bonding and hydrophobic interactions have been revealed. As previously envisaged, aromatic and acidic residues within the binding site (e.g., Trp, Tyr, and Glu) have been found to be the key participants in recognition of the Galα(1,3)Gal epitope. It was also confirmed that conserved Ser and Pro residues, located near the floor of the binding pocket, play a role in binding. *Conclusion.* Despite the enormous clinical potential for xenotransplantation, very little is known about the 3D structural basis for natural antibody recognition of the major carbohydrate xenoantigen. Understanding of structural demands of anti-Gal antibodies with respect to their ligands is necessary for design of successful inhibitors of hyperacute xenograft rejection.

P141
LACK OF N-GLYCOLYLNEURAMINIC ACID IN MONOTREMES AND BIRDS?

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N-Glycolylneuraminic acid (Neu5Gc) is frequently found in the animal kingdom, from echinoderms to higher mammals, but is lacking in man and chicken (1). To answer the question whether birds generally do not express this sialic acid type we investigated tissues and eggs of poultry and birds from different continents. In addition we analysed the milk from the monotreme echidna (Tachyglossus aculeatus) (2) as well as the muscle tissue of the platypus (Ornithorhynchus anatinus), since this ancient branch of egg-laying mammals seems to be anatomically and thus phylogenetically related to the birds and reptiles (Sauropsida).
The sialic acids were liberated by mild acid hydrolysis, purified by ion-exchange chromatography and analysed by TLC and HPLC. In all samples, only N-acetylneuraminic acid (Neu5Ac) and in some probes O-acetylated Neu5Ac were detected. Neu5Gc was also absent from duck liver, although in earlier studies (3) a small amount of this sialic acid had been found in intestine, which may have been originated from food stuffs.
It is unknown why Neu5Gc is lacking in the birds and monotremes studied, therefore molecular genetic experiments are suggested. Evidence for the lack of the gene coding for the CMP-Neu5Ac hydroxylase, which is involved in Neu5Gc biosynthesis, was presented by genomic Southern blot analyses of chicken liver DNA (4).

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P142
THREE-DIMENSIONAL STRUCTURE OF HUMAN *N*-ACETYLGLUCOSAMINE KINASE

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N-acetylglucosamine kinase (GlcNAc kinase, EC 2.7.1.59) converts endogenous GlcNAc from lysosomal degradation or nutritional sources into GlcNAc 6-phosphate. GlcNAc 6-

phosphate then enters the pathway leading to the formation of UDP-GlcNAc, which serves as a substrate of the GlcNAc transferases in oligosaccharide biosynthesis. Furthermore, UDP-GlcNAc is used for formation of intracellular O-GlcNAc modification of proteins and for the biosynthesis of sialic acid. Here we present the crystal structure of human GlcNAc kinase with a resolution of 2.6 A. The complex of GlcNAc kinase with bound ADP and GlcNAc is a dimer which shares common features of other sugar kinases. The catalytic centre exhibits an ATP binding motive[(1)] and our data support an induced fit mechanism during substrate binding which is already described for hexokinase[(2)]. Determination of the kinetic properties of cysteine mutants C131S and C143S of GlcNAc kinase showed that the decreased enzyme activities were due to a strongly decreased affinity of GlcNAc and ATP, respectively[(3)]. These cysteine residues are located in the active site of GlcNAc kinase with a potential role in the binding of the transfered γ-phosphate group of ATP within the catalytic mechanism.

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P143
CELL OVEREXPRESSION OF MAMMALIAN SIALIDASES INHIBITS NEWCASTLE DISEASE VIRUS MEDIATED CELL-CELL FUSION

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Newcastle Disease Virus (NDV) is an avian enveloped virus belonging to the family Paramyxoviridae. Viral membrane contains two glycoproteins: hemagglutin-neuraminidase (HN) and fusion (F) protein. HN binds to sialic acid-containing receptors at the cell surface through its hemagglutinating activity and it also displays sialidase activity. In the sialoglycoconjugate metabolism, a key role is played by sialidases, glycohydrolytic enzymes that cleave sialic acids. The sialic acid content of the cell is tightly regulated by the action of sialidases: Neu3, present into the membrane; and Neu2, in the cytosol. In the present study, we analyzed the effects on NDV-cell interactions of the overexpression of both Neu3 and Neu2 on the target cell. NDV-mediated cell-cell fusion was assessed by a syncytia assay using GIEMSA staining.
Our data revealed that fusion between cells stably expressing human Neu2 and human Neu3, induced by NDV, was reduced 70 % and 27 % respectively, from control. Additionally, cells transfected with the mouse sialidases MmNeu2 and MmNeu3 showed a fusion inhibition of 75 % and 100 %, respectively. The differences in fusion inhibition among both Neu3 expression systems may lay in the level of sialidase expression, 3.7 times higher in transient cells than in stably cells.
We propose that modifications of the sialoglycoconjugate pattern determined by sialidase overexpression provoques changes on the viral receptor availability need for NDV-mediated cell-cell fusion.

P144
ACTIVATION OF PLASMA-MEMBRANE SIALIDASE NEU3 DURING C2C12 MYOBLAST DIFFERENTIATION

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The intricate mechanisms regulating myoblast differentiation are still under investigation. A few reports suggest the involvement of sialidases in muscle differentiation. However, while myotube formation is inhibited by down-regulation of Neu2 (the cytosolic sialidase), nothing has been reported about the role in this process of Neu3 (the plasma-membrane sialidase). Since Neu3 is known to modulate the ganglioside content in the lipid bilayer, we assumed it was involved in the membrane remodelling during myoblast fusion into myotubes. We used a lentiviral vector (pLenti6Neu3/V5) and obtained a C2C12 cell line stably expressing Neu3 to investigate the effect of this protein during differentiation. Unexpectedly, although the levels of Neu3 mRNA in these cells were higher than the controls, and the V5-tagged protein was expressed (as shown by Western blot analysis), no increase of enzymatic activity towards 4-MU-NeuAc and [H^3] GD1a in the undifferentiated myoblast was measured. On the other hand, 2% HS induced differentiation of myoblasts led to a 5 fold increase (8 fold in the presence of 0.1% TRITON X-100) of the activity towards GD1a. These preliminary results suggest that Neu3 activity is important in the differentiation stage. In proliferating cells Neu3 may be confined to an as yet unknown compartment and therefore unable to cleave the substrates, alternatively enzyme activity may be strictly regulated in a currently unknown way. The mechanisms of Neu3 activity regulation are under current investigation.

P145
DISCOVERY OF POTENT AND SELECTIVE α-FUCOSIDASE INHIBITORS

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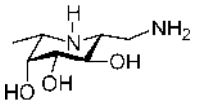
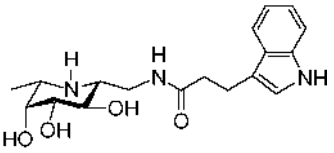
Glycosidases represent an important class of enzymes as therapeutic targets, as exemplified by Tamiflu™, a well-known neuraminidase inhibitor to treat the infection of influenza virus. Miglitol™ is another good example targeting the intestinal disaccharidases and is currently prescribed for insulin-independent diabetes. Herein we report the discovery of picomolar, slow tight-binding inhibitors against the α-fucosidase from *Corynebacterium sp.* (Csfd) and *Thermotoga maritima* (Tmfd) by a rapid screening for an optimal aglycon attached to 1-aminomethyl fuconojirimycin **1**. The introduction of a hydrophobic moiety not only resulted in the time-dependent inhibition, but also greatly enhanced the inhibitory potency from a low nano molar K_i to picomolar K_i* value. Compound **2** with a K_i* of 0.46 pM against Csfd represents the most potent glycosidase inhibitor to date. The effect of inhibitor titration with compounds on the intrinsic fluorescence is both time- and concentration- dependent, in good consistence with the measured enzyme kinetics. Additionally, various substituted fuconojirimycins have been prepared, including the substitution at C1, C2, C6 and N-positions, to give a diversity of more than 240 compounds. Their inhibition was then studied and compared to disclose the possible extra binding site to enhance the affinity.



1



2

P146
CHARACTERIZATION OF THE MURINE MEMBRANE-ASSOCIATED SIALIDASE NEU4

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NEU4 is the fourth member of the sialidase gene family cloned so far and encodes for a membrane associated enzyme with an acidic pH optimum. Two isoforms of the protein have been described in human: the long isoform is characterized by a 12 amino acid sequence at the N-terminus that is missing in the short form of the protein. The long form of NEU4 localizes to mitochondria or lysosomes, while the short form localizes to intracellular membranes. Aim of this work is the characterization of *Mus musculus* NEU4. Interestingly, for this protein only the short isoform has been detected. The cDNA encoding *Mm*NEU4 has been cloned into the pcDNA3.1/myc-His plasmid and transiently expressed in COS7 cells. By confocal microscopy analysis the protein was found to be associated to a membranous network that surrounds the nuclear membrane and mitochondria but no precise colocalization with any cellular marker could be demonstrated. Administration of radiolabelled ganglioside GD1a to NEU4 transfected cells in conditions that prevent lysosomal activities led to its hydrolysis into ganglioside GM1, indicating that, although most of the protein is associated to the inner cell membranes, a significant amount is also present at the cell surface. Using the rough particulate fractions obtained after 100,000*g* ultracentrifugation as enzyme source, NEU4 activity was characterized using both the artificial substrate 4MU-NeuAc and tritiated GD1a ganglioside. The enzyme showed an extremely acidic optimal pH (3.2) and hydrolyzed GD1a ganglioside only in the presence of 0.1% Triton X-100.

P147
NEU3 OVEREXPRESSION MODIFIES THE GANGLIOSIDE PATTERN OF LIPID RAFTS

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The plasma membrane-associated sialidase NEU3 is involved in the regulation of cell surface sialic acid levels. The enzyme is associated with caveolin in sphingolipids enriched domains (SED) and its ability to modify the ganglioside pattern of neighboring cells has been recently demonstrated. In order to investigate its possible role in regulating the SED lipid composition, COS7 cells were transiently transfected with the *Mus musculus* NEU3 cDNA (*Mm*NEU3), carrying a C-terminus haemoagglutinin epitope (HA). Sphingolipids were metabolically labelled with [1-^{3}H]-sphingosine and SED isolation was carried out using an OptiPrep discontinuous density gradient. Caveolin-enriched low density fractions contained roughly 40% of *Mm*NEU3 as detected with Western-blot technique and showed a 5.5 fold increase in specific activity compared with the high density fractions. As already reported for typical SED preparations, low density fractions contained approximately 50% of the total cell gangliosides. After 48 hours *Mm*NEU3-expressing cells showed a decreased content of gangliosides GD1a and GM3 of about 65 and 50% respectively, compared to mock-transfected cells. At the same time, a 100% increase of GM1ganglioside was observed. Overall, these results demonstrate that *Mm*NEU3 overexpression specifically modifies the SED ganglioside pattern. Further experiments are

in progress in order to characterize the possible activity of the enzyme on neighboring cell SED.

P148
ATYPICAL KINETIC BEHAVIOUR OF HYALURONIDASE AT HIGH HYALURONAN CONCENTRATIONS AND UNDER LOW IONIC STRENGTH CONDITIONS

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Hyaluronan is an anionic polysaccharide of high molecular weight composed of N-acetyl-D-glucosamine and D-glucuronic acid alternatively linked through β(1-4) and β(1-3) glycosidic bonds. Hyaluronidases of the testicular type catalyse the hydrolysis of β(1-4) glycosidic bonds of hyaluronan. It appears that i) high levels of both hyaluronan and testicular type hyaluronidase are found in tumours and ii) contrary to high molecular weight hyaluronan, oligosaccharides stimulate angiogenesis. As cancer tumour growth and metastasis are dependent upon vascularisation, the control of the balance between high molecular weight hyaluronan and oligosaccharides through the hyaluronidase activity is supposed to play an important role in cancer development. It is thus fundamental to study the kinetics of that enzymatic reaction.
Initial hyaluronan hydrolysis rate were determined by using our variant the Reissig method which estimates the reducing ends formed. While the substrate-dependence obtained in the presence of 150 $mmol.L^{-1}$ ionic strength exhibits a Michaelian type behaviour, it shows an atypical shape in the presence of a very low ionic strength (5 $mmol.L^{-1}$) : for increasing hyaluronan concentrations, the initial rate increased, reached a maximum and then decreased down to a very low level.
To explain those results, several hypotheses, such as “salting in” – “salting out” phenomena, high viscosity and low water activity of concentrated hyaluronan solutions and existence of non specific and non catalytic hyaluronan – hyaluronidase complexes, were examined. It results that i) the most pertinent explanation concerns the existence of hyaluronan – hyaluronidase complexes and ii) diffusion is not the kinetic limiting factor.

P149
STUDY OF BIOSYNTHESIS OF ACIDIC EXOPOLYSACCHARIDE IN MUTANT CELLS OF RHIZOBIUM LEGUMINOSARUM BV. VICIAE VF39

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The Rhizobium leguminosarum bv. viciae VF39 synthesize an acidic exopolysacharide (EPS), which is necessary to establish nitrogen fixing symbiosis with Pisum sativum, Vicia faba, and Vicia sativa. This EPS is a polymer of an octasaccharide repeating unit with acetyl, pyruvyl and hydroxybutanoyl modifications [1]. Biosynthesis of this type of polysaccharide chains is usually proceeds through polyprenyl phosphate intermediates containing repeating oligosaccharide moiety. Gene cluster responsible for octasaccharide repeating unit assembly was sequenced and mutants deficient in some of these genes were prepared [2]. Results of biochemical experiments and structural analysis of EPS produced by mutant cells allow to propose enzymic functions to products of some genes of the cluster.
Initiation of the octasaccharide biosynthesis is catalysed by UDP-Glc: polyprenyl-phosphate glucosephosphotransferase encoded by pssA gene. Synthesis of [^{14}C]Glc-ppPre was confirmed by TLC. PssA mutants were unable to start the EPS biosynthesis. It was shown the pssD and pssE genes to encode the second step of octasaccharide fragment assembly, namely transfer of GlcA residue on C-4 of Glc-ppPre. Functions for other genes of cluster, pssC, pssF, pssG, pssH and pssI, were not determined as all cells with mutations on these genes were able to synthesize EPS characterized by the same structure as wild type EPS as was confirmed by NMR analysis.

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P150
CLONING, EXPRESSION AND ER RETENTION OF MAMMALIAN FATTY ACID-2-HYDROXYLASE

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Fatty acid-2-hydroxylation is an abundant modification of galactocerebroside (GalC) and sulfatide in the mammalian brain. Fatty acid-2-hydroxylated cerebrosides are the most abundant sphingolipids in the myelin sheath. The functional role of this modification, however, is not known. Recently, we and others identified murine and human cDNAs with a significant similarity to the yeast C26 fatty acid-2-hydroxylase (FA2H) gene. Transfection of the putative murine FA2H cDNA in CHO cells led to the formation of hydroxylated fatty

acid containing hexosylceramides. Furthermore, FA2H expression is up-regulated during the period of active myelination, when the synthesis of 2-hydroxylated cerebroside and sulfatide are increased. These data strongly suggest that FA2H is the enzyme responsible for the formation of hydroxylated sphingolipids in myelin. Expression of FA2H in UDP-galactose:ceramide galactosyltransferase positive CHO cells led to a significant up-regulation of total GalC level and down-regulation of long chain fatty acid containing sphingomyelin. These data clearly show that the expression level of FA2H significantly affects the steady state levels of various sphingolipids and that the steady state levels of these lipids is not only regulated by the activity of their synthesizing enzymes. An epitope-tagged FA2H proteins co-localized with calnexin indicating that the enzyme resides in the endoplasmic reticulum (ER). FA2H contains a potential carboxy-terminal ER retrieval motif (His-X-Lys-X-X). Exchanging the lysine residue by leucine shifted the enzyme to the Golgi apparatus, indicating that the ER retrieval motif is functional.

P151
HUMAN PLASMA MEMBRANE ASSOCIATED ß-HEXOSAMINIDASE A HYDROLIZES GM2 GANGLIOSIDE

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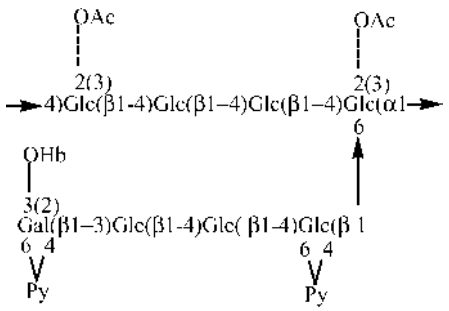
Plasma membrane (PM) glycosphingolipids (GSLs) are modulators of signal transduction processes involved in cell proliferation, survival and differentiation events. Their changes during development and neoplastic transformation suggest a role as receptor site in cell-cell recognition. Glycosyl epitopes change includes many different residues, such as GlcNAc, GalNAc in N-linked structure or sialyl residues in either N-linked, O-linked or lipid-linked structure, so that cell surface glycohydrolases could be one of the natural candidate for PM glycosylation modification.



Here we demonstrate the occurrence on PM of β-hexosaminidase A, an acidic glycohydrolase that cleaves terminal β-linked GlcNAc or GalNAc residues from oligosaccharides, glycolipids, glycoproteins and glycosaminoglycans and hydrolizes ganglioside GM2, in combination with a specific activator protein. Separation of PM associated β-hexosaminidase A from the intracellular counterparts was achieved by *in vivo* cell surface biotinylation followed by affinity chromatography purification of the biotinylated proteins, and by immunocytochemistry. The immunological and biochemical characterizations of β-hexosaminidase revealed that the PM associated enzyme is fully processed, indicating its lysosomal origin and excluding in transit precursors. Moreover by an *in vitro* assay we found that cell surface β-hexosaminidase A is enzymatically active towards the natural substrate GM2 ganglioside in the presence of GM2 activator protein.
These findings lay the basis for future study exploring cell surface β-hexosaminidase potential involvement in GSL oligosaccharide modification processes, regulating cell-to-cell and/or cell-environment interactions in both physiological and pathological mechanisms.
Acknowledgments:
Work supported by COFIN-PRIN and FIRB grants to S.S., C.E., A.O.

P152
EXPRESSION OF LYSOSOMAL PROTEASES CATHEPSIN D, B AND L IN FIBROBLASTS FROM ALZHEIMER'S DISEASE PATIENTS

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Previous studies established that the population of neurons that frequently degenerate in Alzheimer's Disease (AD) exhibit robust up-regulation of the lysosomal system. We investigated the expression of lysosomal proteases cathepsin D, B and L at peripheral level, using as cell model skin fibroblasts from AD patients affected either by sporadic or familial forms of the disease. By enzymatic assays, we observed a down-regulation of cathepsin D in 50% of AD patients. These decreased levels were consequent to regulation at transcriptional level. Western Blotting analysis confirmed the presence of decreased content of cathepsin D in AD

fibroblasts. We extended the analysis to cathepsin B and cathepsin L and observed decreased content of these proteins in the same cells. A parallel increase of ras transcript and ras protein had been previously revealed in AD fibroblasts. To investigate the role of ras in the expression of cathepsin D, B and L, we over-expressed in primary fibroblasts a ras mutant (rasV12) known to induce premature senescence. Fibroblasts infection with a retrovirus encoding a constitutively active ras decreased cathepsin D levels but increased levels of cathepsin L and, to a lesser extent, of cathepsin B. Decreased cathepsin levels and activities in AD could lead to a decreased ability of proteolysis by the cell and might be related to the pathogenesis of the disease.

Acknowledgements
Work supported by PRIN-Cofin and FIRB grants to C.E.and A.O.

P153
EVIDENCE FOR ALTERED LATE ENDOSOMAL-LYSOSOMAL COMPARTMENT IN SANDHOFF FIBROBLASTS

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We are characterizing the endosomal-lysosomal compartment of Sandhoff fibroblasts in order to understand the molecular mechanism underlying the altered endocytic trafficking observed in many lysosomal storage diseases.
Sandhoff disease is a GM2 gangliosidoses due to mutations in the gene encoding the β- subunit of β-hexosaminidase.
We analyzed endosome/lysosome distribution in a density gradient of a microsomal fraction from Sandhoff and normal fibroblasts. Densitometric analysis of Western blotting of a 0.5-1.6 M continuous sucrose gradient fractionation showed that late endosomes, detected using anti-Rab7 antibodies, were mainly present at the control fractions 4 and 9-10 and at the Sandhoff fibroblast fractions 6 and 10; lysosomes, detected using anti-LAMP2, resulted mainly distributed in the light fraction 3 in both cell types and in a dense fraction corresponding to the 11 for control and the 15 for Sandhoff fibroblasts. The density profile of early endosomes, detected using anti-Rab5A antibodies, resulted very similar in both cell types, presenting a diffused distribution in the fraction range 4-8.
Fluorescence microscope measurements using the FITC-dextran marker, showed that Sandhoff fibroblast vesicles appeared brighter and prevalently localized in a restricted perinuclear areas compared to the characteristic control cell punctate distribution. Lysosomal pH, measured using the pH-sensitive FITC-dextran probe, resulted to be 5.03 in both cell types. Therefore the higher fluorescence of Sandhoff fibroblast vesicles wasn't due to differences in lysosomal pH.
These results suggest that the traffic alteration probably arise in the last steps of endocytic pathway.
Work supported by FIRB and PRIN-Cofin.

P154
IGF-I INDUCED HYPERTROPHY OF MYOBLASTS INCREASES CYTOSOLIC SIALIDASE NEU2 THROUGH PI-3 KINASE/AKT/MTOR PATHWAY.

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Cytosolic sialidase Neu2 has been implicated in myoblast differentiation (1). Neu2 transcript and enzymatic activity are strongly up-regulated during myotubes formation and Neu2 overexpression in C2C12 myoblasts enhances the differentiation through a marked cell hypertrophy (2).
Here we describe that in myoblasts Neu2 enzymatic activity is strictly dependent from the Igf-I signalling. Igf-I has been shown to be sufficient to induce differentiation and hypertrophy of muscle cells through either autocrine or paracrine mechanisms (3). We observed during Igf-I induced hypertrophy of C2C12 cells a remarkable increase of Neu2 enzymatic activity dependent from the activation of PI-3 kinase/Akt/mTor pathway. The dependence of Neu2 activity from Igf-I signalling has been confirmed using C2BP5 myoblasts (4), a cell line overexpressing IGF binding protein-5 (IGFBP-5) that cannot differentiate in response to endogenously produced Igf-I. The treatment of these cells with R3-Igf-I, analog form of Igf-I which is not bound by the IGFBP-5, promotes differentiation restoring Neu2 enzymatic activity. According to these results, Neu2 overexpression induces myoblast hypertrophy, suggesting a relevant role for this sialidase in this process.
Finally, we established that myoblasts overexpressing Neu2 undergo to hypertrophy even when treated with a chemical inhibitor of gangliosides biosynthesis, suggesting that presumably gangliosides are not substrates for Neu2 activity during myoblast differentiation .

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P155
IDENTIFICATION OF THE STRUCTURAL UNITS INVOLVED IN DIFFERENTIAL RECOGNITION OF NEU5AC AND KDN IN THE VERTEBRATE CMP-SIA SYNTHETASES

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A CMP-Sia synthetase (CSS) synthesizes CMP-Sia from CTP and Sia. The mouse, human, and rainbow trout CSSs were recently cloned. They are composed of 432 amino acids, and the catalytic domain exits in N-terminal half of the enzyme. There are five conserved amino acid sequence motifs (motifs I-V) among these CSSs. The mouse CSS exhibits a preferential specificity for *N*-acetylneuraminic acid (Neu5Ac) to deaminoneuraminic acid (KDN), while the rainbow trout CSS is equally active with either of these Sia species. However, it has remained unknown how the CSSs recognize the Sia species at the molecular level. Thus, an objective of this study is to identify structural elements for the recognition of Sia species. We analyzed the chimeric and site-directed mutants of the mouse and rainbow trout CSSs for the *in vitro* activity toward Neu5Ac and KDN using the recently developed assay [Fujita *et al.* (2005) *Anal. Biochem.* 337, 12]. We prepared and analyzed more than 40 chimeric mutants of the mouse and rainbow trout CSSs. We showed that the chimeric enzyme in which the motif IV-V region of the rainbow trout CSS was substituted for the corresponding region of mouse CSS had the similar activity to the rainbow trout CSS, although the activity became low. A few enzymes of site-directed mutations in this region lost their CSS activity completely. These results indicate that this region is involved in the enzyme activity as well as in differential recognition of Neu5Ac and KDN.

P156
A BIOINFORMATIC APPROACH TOWARDS THE ELUCIDATION OF MEMBRANE ANCHORAGE OF NEU3 AND NEU4 HUMAN SIALIDASES

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Crystal structure of human cytosolic sialidase NEU2 has recently been elucidated. It shows the canonical six blades beta-propeller observed in viral and bacterial sialidases, with its active site in a shallow crevice. On the other hand, nothing is known so far about the structure of two other members of the protein family: NEU3 and NEU4 sialidases. Although the three enzymes show a high homology in their primary structures, NEU3 and NEU4, unlike NEU2, are membrane associated.

In order to get some insight into the mechanisms through which these proteins become associated to the membranes, we undertook a bioinformatic study on NEU3 and NEU4 sequences and on their predicted tertiary structures. A similar approach had been usefully exploited in the past for the elucidation of lysosomal sialidase NEU1 threedimensional structure.
Analysis of aminoacidic sequences showed the absence, both in NEU3 and NEU4, of myristylation, palmitoylation or GPI anchor motives. Hydrophobicity plot showed NEU3 to be the most hydrophobic sialidase, while NEU4 is only slightly less hydrophilic than NEU2, but failed to show any typical transmembrane domain. Prediction of NEU3 and NEU4 tertiary structure showed, as expected, that both these proteins share with NEU2 the basic six blades beta-propeller. Nevertheless a hydrophobic loop connecting two beta-strands was found to be present in both membrane associated enzymes, but absent in the cytosolic sialidase NEU2.
We propose that these loops might be involved in NEU3 and NEU4 membrane anchorage. Site-directed mutagenesis will be performed accordingly in order to validate this hypothesis.

P157
ISOENZYME PATTERN AND PARTIAL CHARACTERIZATION OF HEXOSAMINIDASES IN HUMAN ERYTHROCYTES.

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Introduction. Hexosaminidase (Hex) is present in lysosomes, plasma membrane and cytosol of many human cells. Whereas lysosomal Hex is well characterized in its subunits composition of isoenzymes, coding genes and role in lysosomes, there are little information about membrane and cytosolic ones.
Experimental approaches. Our objective is, utilizing different techniques, characterize the erythrocytes Hex isoforms and their subunit components in respect to those present in plasma of controls, pregnant women and in a Tay-Sachs subject.
Results. In plasma membrane and in cytosol Hex presents two group of isoenzymes that, for their characteristics, are composed by the same subunits of plasma Hex A and B isoenzymes. The Hexs in erythrocytes and those present in lysosomes have a common genetic origin and α- and ß-subunits association occurs in the same way, then the membrane and cytosolic localization of these isoenzymes is probably due to unknown posttranslational modifications. Noteworthy, in cytosol there is an Hex that for its characteristics is a specific N-acetyl-ß-D-glucosaminidase probably the same well known specific for the hydrolysis of N-acetylglucosamine residues O-linked to proteins.
Conclusions. The study of isoenzymatic pattern in specific physiological and pathological conditions offers an attempt to

explain the origin and subunit composition of the different forms and to evaluate their relationships with the plasmatic and cellular ones. Experiments are presently underway to purify the different isoenzymes, in particular the cytosolic GlcNAc-ase for its characterization with regard to the subunit composition.

P158
LYSOSOMAL LEUKOCYTE ß-D-GLUCURONIDASE DURING ENZYME REPLACEMENT THERAPY IN FABRY DISEASE

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Introduction. Fabry disease results from a deficiency in the activity of α-D-galactosidase A and subsequent accumulation of neutral glycosphingolipids in lysosomes. Till now, it is still difficult to find a good biochemical parameter for monitoring the outcome of enzyme replacement therapy (ERT). This study investigated whether lysosomal enzymes can indicate biochemical changes in the lysosomal apparatus induced by ERT.
Experimental approaches. Eight patients, (six males, aged 26-43 years, and two females, aged 48 and 54 years) treated with ga-haGalA (0.2 mg/kg b.w. i.v. every two weeks, Replagal, TKT) or r-haGalA (1 mg/kg b.w. i.v. every two weeks, Fabrazyme, Genzyme) were monitored by clinical and biochemical tests and several lysosomal glycohydrolases were measured in plasma and leukocytes.
Results. Before starting ERT, ß-D-glucuronidase in leukocytes was markedly increased. After one month of therapy, enzyme levels dropped in all patients. In the patients who regularly followed the therapy, the enzyme levels remained stable for the next 20 months. In one patient who interrupted therapy for two months, the enzyme levels rose again.
Conclusions. Lysosomal enzymes can be useful for monitoring biochemical changes in patients with Fabry disease receiving ERT. Though these findings refer to only a small number of patients, the correlation between ß-D-glucuronidase levels and ERT is interesting and might serve as a basis for further studies to define the potential of this enzyme in monitoring the effects of ERT in lysosomal storage disorders.

P159
THE ALTERNATIVELY SPLICED ST3Gal III-A1, -B1 AND -C1 ISOFORMS SHOW ENZYMATIC ACTIVITIES BUT ARE DIFFERENTLY DISTRIBUTED WITHIN THE CELL.

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The human α2,3-sialyltransferase III, ST3Gal III, is known to transfer sialic acid to the type 1 chain precursor (Galβ1,3GlcNAcβ). The ST3Gal III gene has been shown to generate 26 alternatively spliced RNA transcripts of the coding region with a wide variety of deletions and insertions. The features of the transcripts revealed a systematic pattern and the transcripts were classified into five main isoforms (A-E) each with up to twelve subgroups (1-12).
ST3Gal III-A1, -B1 and C1 all display identical N-terminals, transmembrane regions and C-terminal catalytic regions with the three sialylmotifs intact and with differences only in the stem region. In order to investigate the biological consequences of the variations in this region of the human ST3Gal III enzyme, we established stably transfected human embryonic kidney cells (293-cells) expressing both unlabeled and Myc-labeled A1, B1 and C1 isoenzymes. Crude cell extracts from these cell lines were used for kinetic studies and for subcellular fractionations.
Our data revealed that all three isoenzymes exhibited enzymatic activities preferentially towards the type 1 chain precursor although with different kinetics. The subcellular fractionation revealed that all three isoenzymes were identified both in ER and Golgi fractions, but with different patterns of distribution. After glycosylation A1 and B1 isoenzymes were only localized within the Golgi, whereas the C1 isoenzyme was partially recycled back to ER.

P160
GLYCOSIDASES OF *CAENORHABDITIS ELEGANS* INVOLVED IN N-GLYCAN PROCESSING

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Recent data indicates that in addition to the Golgi α-mannosidases, the model nematode *Caenorhabditis elegans*

also possesses, like insects, an *N*-acetylhexosaminidase activity putatively involved in N-glycan processing in the Golgi. The presence of such an activity is invoked, not just on the basis of the detected enzyme activity, but also to explain the absence of terminal *N*-acetylglucosamine residues on structures which require the prior action of *N*-acetylglucosaminyltransferase I during their biosynthesis. In order to understand the genetic basis for these activities, we have cloned cDNAs encoding members of both glycohydrolase families 20 and 38 from the worm. The encoded glycosidases were expressed in the yeast *Pichia pastoris* as soluble forms lacking putative cytoplasmic and transmembrane domains. Four glycohydrolase family 20 members were shown to cleave *p*-nitrophenyl-β-*N*-acetyl-glucosaminide and/or *p*-nitrophenyl-β-*N*-acetylgalactosaminide, but showed contrasting specificities with regard to N-glycan substrates. On the other hand, one glycohydrolase family 38 member was shown to be active using *p*-nitrophenyl-α-mannoside as a substrate and, in addition, had mannosidase II activity. These, therefore, are the first data on the activity of *Caenorhabditis* glycosidases towards N-glycan substrates and should aid the further elucidation of N-glycan processing in this organism.

P160b
CLONING, RECOMBINANT EXPRESSION AND ENZYMATIC ACTIVITY OF A TRANS-SIALIDASE FROM *TRYPANOSOMA CONGOLENSE*

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Trypanosoma congolense is a livestock-infective trypanosome causing Nagana disease. Similar to other pathogenic trypanosomes, *T. congolense* expresses a trans-sialidase (TS) transfering sialic acids from sialoglycoconjugates to β-galactosides. Two TS forms have been purified (Tiralongo et al. 2003a) and fragments of two candidate genes have been described (Tiralongo et al. 2003b). However, the full length sequence of an active enzyme has been unknown.
Using these partial TS sequences as query sequences, four TS-like genes were identified from the shotgun sequencing project of the *T. congolense* genome at The Wellcome Trust Sanger Institute. Two of these are previously unknown TS-like genes, whereas the other two represent the full lenght sequences of the gene fragments described (Tiralongo et al. 2003b). One of these (TS1) was amplified from genomic DNA and expressed in *E. coli*. The purified recombinant protein was then shown to exhibit TS activity.
It is most likely that TS1 is the active TS produced by *T. congolense*, since it contains the sequence of a peptide obtained from an active form of the enzyme purified from *T. congolense* cultures and has similar kinetic parameters.
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P161
A NOVEL LYSOZYME WITH SERINE PROTEASE INHIBITOR HOMOLOGY FROM THE SOLDIER DEFENSE GLAND OF THE FORMOSAN SUBTERRANEAN TERMITE: *COPTOTERMES FORMOSANUS*

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Coptotermes formosanus, the Formosan Subterranean Termite (FST), was imported to the Southern USA between 1940-50, and has spread throughout Louisiana and surrounding states destroying wooden structures and invading forests where the local trees have no defense. Many Live Oak trees in some areas of New Orleans are infested, as well as many old buildings in the French Quarter and other areas. Soldiers comprise ~20% of the nest, and are fed by trophallaxis from the workers, having mandibles modified for defense. The frontal defense gland comprises 1/3 of the body mass of the FST soldier and begins as a cavity in the head extending into the body. A foramen in the head is used for discharging the defense fluid on attacking predators such as ants. This gland comprises 7% of the biomass nest, suggesting an important evolutionary history, and is a significant nutrient expenditure. The secretion stimulates transformation of workers to soldiers in subsequent molts after exposure to the defense fluid, recruits soldiers and repels workers from the area of danger. It is interesting that the products of a defense reaction which may result in the loss of soldiers, stimulates production of new soldiers during subsequent molts of exposed workers. Little is known about the function of any component of the defense

secretion, however. We have identified a number of small molecules in the secretion, such as ceramides and free fatty acids, and as many as 9 new proteins. By using a zymogram gel, we have found, cloned and expressed a novel, low molecular weight lysozyme that has no homology to known lysozymes in the Drosophila genome, nor to 2 other putative lysozyme genes we cloned in the FST genome. Instead, the unusual 6400da lysozyme, which apparently functions as a dimer, has homology to serine protease inhibitors. We postulate that this lysozyme may act as an antibiotic in the FST defense fluid. Some other defense proteins (perhaps with participation of some small components) may also participate to form an immobilization glue that impedes invaders.

P162
C-TERMINAL TRUNCATION OF HELICOBACTER PYLORI 1,3-FUCOSYLTRANSFERASE TO IMPROVE PROTEIN PRODUCTION, SOLUBILITY AND ACTIVITY

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As an important human pathogen to cause gastric and duodenal ulcers, Helicobacter pylori is the major cause of gastric cancer and lymphoma. This pathogen can express fucose-containing Lewis x and Lewis y antigens to mimic the cell-surface glycoconjugates of gastric epithelium cells. Such mimicry is considered to prevent the surveillance of host immune system. We have cloned the H. pylori α1,3-fucosyltransferase (FucT) of 478 amino acid residues. Wild type FucT was expressed in E. coli at high level but in an insoluble form. In order to improve the insolubility, several mutants of different size were prepared by C-terminal truncation. The results indicated that up to 45 amino acid residues, the more residues were deleted, the more soluble proteins were obtained. Up to 80 residues, the truncated FucTs catalyzed the fucose transfer from GDP-fucose (donor) to N-acetyllactosamine (acceptor). However, further deletion (C-terminal 115 residues) not only resulted in 10- and 3-fold higher K_m values of acceptor and donor substrates, respectively, but also dramatically changed the quaternary structure.

P163
PROTEOMIC MAPPING OF FUNCTIONALLY IMPLICATED GLYCOSYLTRANSFERASE ACTIVITIES AS AN ESSENTIAL COMPENDIUM TO GLYCOMIC PROFILING OF TUMOR-ASSOCIATED ABERRANT GLYCOSYLATION.

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Aberrant glycosylation is a common phenotype of carcinomas. Among various tumor associated glycotopes, extended type 1 chain in the form of Lewis$^{b/a}$-Lewis^{a} has been identified in the glycosphingolipids of human colonic adenocarcinoma cell line Colo205. Adopting this as a model system to develop enabling glycomic methodologies, we have since demonstrated the presence of terminal Le^{a}-Le^{a} epitope on both N- and O-glycans. On one hand, mass spectrometry (MS)-based techniques are being employed to further localize the glycotopes on specific glycoprotein carriers. On the other hand, proteomics analyses are undertaken to identify the most abundantly expressed or abnormally up-regulated glycosyltransferases which may account for the synthesis of the implicated glycotopes. Mapping at the protein expression level in concert with enzyme activity assay is proposed herein to be an essential compendium to glycomic profiling, in addition to the more commonly performed transcriptomic mapping of the glycosyltransferase genes by northern blot, RT-PCR, and/or qPCR at mRNA level. We report here our one-step enrichment strategy employing GDP affinity column which led to successful proteomic ID of FucT III, FucT VI and OFUT1, among the most abundantly expressed FucT in Colo205. MS analysis of endogenous FucT allows a meaningful dissection of its post-translational modifications in relation to its functional specificity. In parallel, a novel FucT activity assay based on the measurement of in-solution proximity binding with photosensitizers was successfully developed. A preferred synthesis of Le^{a} over Le^{x} was demonstrated by the endogenous FucT activities ensemble when assayed against type 1 and 2 lacNAc acceptor unit.

P164
SLOW-BINDING INHIBITION OF 1-SUBSTITUTED FUCONOJIRIMYCINS AGAINST THERMOTOGA MARITIMA α-L-FUCOSIDASE

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α-L-Fucosidases are enzymes to hydrolyz the α-fucosidic linkage to release L-fucose from glycoconjugates and usually participate a variety of important biological processes. To study their functions and develop therapeutic agents relies on the discovery of potent fucosidase inhibitors. We recently have developed the rapid synthesis of fuconojirimycin-derived inhibitors in microtilter plates and in situ high-throughput

screening established to identifiy a serious of potent and selective inhibitors against the α-L-fucosidases from Thermotoga maritima and human tissue. Interestingly, slow-tight binding inhibition was found in the study of the former enzyme, distinct from the reversible inhibition of the latter. According to computational modeling, the drastic difference in the inhibition was probably attributed to the hydrophobic aglycon binding site that was absent in human fucosidase. To decipher the interesting distinction at molecular level, several residues likely involved in the aglycon binding were changed to alanine by site-directed mutagenesis. Slow-binding inhibition was diminished to a different degree in the activity assays of these mutants. Futhermore, some mutants nearly had no change of the intrinsic fluorescence upon inhibitor titration, in consistance with the aforementioned no time-dependent inhibition, indicating that these mutants behaved like their human counterpart. The result not only agrees to the corresponding kinetic data, but also corroborates the role of the proposed hydrophobic residues in the aglycon binding site.

P165
MECHANISM AND ENERGETICS OF SUBSTRATE BINDING AND CATALYSIS BY CLASS 1 (GLYCOSYLHYDROLASE FAMILY 47) α-MANNOSIDASES INVOLVED IN N-GLYCAN PROCESSING AND ENDOPLASMIC RETICULUM QUALITY CONTROL

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ER quality control determines the fate of newly synthesized glycoproteins toward either correct folding or ER-associated degradation. Initiation of the disposal process involves selective trimming of N-glycans attached to misfolded glycoproteins by ERManI and recognition by the ER-localized EDEM family of lectins, both members of glycosylhydrolase family 47 (GH47). The hydrolytic mechanism catalyzed by members of this family and the energetics of substrate binding is investigated here by a combination of kinetic and binding analyses of wild type and mutant forms of human ERManI as well as by structural analysis of a co-complex with an uncleaved thiodisaccharide substrate analog. These data reveal the roles of catalytic acid and base residues and identify a novel $^{3}S_{1}$ sugar conformation for the bound substrate analog. The co-crystal structure described here, in combination with the $^{1}C_{4}$; conformation of a previously identified 1-deoxymannojirimycin co-complex, indicates that glycoside bond cleavage proceeds through a least motion conformational twist of a properly predisposed substrate in the -1 subsite. A novel $^{3}H_{4}$ conformation is proposed as the exploded transition state. The contributions of several amino acid residues and an enzyme-associated Ca^{2+} ion to substrate binding and catalysis were also demonstrated. One mutant resulted in an enzyme that possessed increased glycan binding affinity, but compromised glycan hydrolysis. A series of glycan binding studies were also performed with this mutant to examine the energetics of substrate binding and to provide a framework for studies on enzyme-substrate interactions for the GH47 enzymes. (Supported by NIH grants GM47533 and RR05351)

P166
CLONING AND EXPRESSION OF A NOVEL MANNOSYLTRANSFERASE GENE CANDIDATE, HMAT-XA, THAT IS HOMOLOGOUS TO DROSOPHILA GENE ENCODING Q9VXN0 PROTEIN.

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Among 14 genes involved in the synthesis of lipid-linked oligosaccharide, the genes encoding mannosyltransferases (Man-T) II and IV have not yet been cloned. In an attempt to find candidates for these genes, we have cloned a novel human Man-T gene, tentatively designated as Hmat-Xa, that is homologous to Drosophila gene encoding Q9VXN0 protein with the glycosyltransferase group 1 domain. Hmat-Xa is also homologous to human genes Hmat-3 (encoding Man-T III) and Hmat-5 (encoding Man-T V), and two bacterial Man-T genes. Whereas the human Man-T I and Man-T III genes are expressed ubiquitously in all tissues, the Hmat-Xa gene shows a tissue-specific expression. Moreover, although the biosynthetic pathway for lipid-linked oligosaccharide is highly conserved among eukaryotes, there is no yeast homolog to the Hmat-Xa gene. Therefore the Hmat-Xa gene may not be involved in the synthesis of lipid-linked oligosaccharide.
We constructed the HA-tagged Hmat-Xa gene in the pMH vector and transfected the construct into HeLa cells. The expression of the transfected gene was confirmed by PCR, RT-PCR, Western blotting and immunoprecipitation. The 50.8 kDa product was detected as the HA-tagged Hmat-Xa protein. The transfected HeLa cells showed a several-fold increase in transcription of Hmat-Xa probably due to expression of the transfected HA-tagged Hmat-Xa gene. The transfected cells also showed altered lectin sensitivities. Therefore it is likely that the Hmat-Xa protein is a glycosyltransferase.

P167
SYNTHESIS OF 6'-SULFODISACCHARIDES BY *ASPERGILLUS ORYZAE* β-*N*-ACETYLHEXOSAMINIDASE-CATALYZED TRANSGLYCOSYLATION REACTION

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Sulfated cell-surface oligosaccharides are widely distributed in glycolipids and glycoproteins as well as glycosaminoglycans. They serve as bio-informational molecules related to neural cell migration, bacterial and viral infections, and selectin bindings. Therefore, facile and practical synthesis of these sulfated sugars has been great subject. In our preceding study, we have demonstrated the high synthetic potential of *N*-acetylhexosaminidase (β-NAHase) for regioselective transglycosylation.[1] There, we found that the presence of 2,*N*-acetyl and allyl groups at the aglycon of acceptors tends to enhance the enzyme-catalyzed transglycosylation yields. In this report, we synthesized various Glc or GlcNAc glycosyl acceptors carrying methyl (CH_3), propyl ($CH_2CH_2CH_3$), allyl ($CH_2CH{=}CH_2$) and *p*-nitrophenyl (*p*NP; C_6H_4-NO_2) groups at the anomeric positions for the examination of the transglycosylation yields. Notable differences were observed between the aglycon structures and the transglycosylation yields. The enzyme highly transferred 6-sulfo GlcNAc moiety to 4-OH of the acceptors having electron-dense glycosides in the order of *p*NP > allyl > propyl > OH > methyl. GlcNAc acceptors always enhanced in the yields compared to Glc acceptors. *p*NP GlcNAc acceptor was an excellent substrate which can be easily applicable to glycosyl chips or arrays after being converted to *p*-aminophenyl group.[2]

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P168
IN VIVO COMPARISON OF THE NCAM POLYSIALYLATION COMPETENCE OF THE TWO POLYSIALYLTRANSFERASES ST8SIAII AND ST8SIAIV

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Introduction. Polysialic acid (polySia), a dynamically regulated posttranslational modification of the neural cell adhesion molecule NCAM, plays a vital role in neural development and plasticity. The biosynthesis of this unique carbohydrate polymer is catalyzed by two polysialyltransferases, ST8SiaII and ST8SiaIV.
Experimental approach. To dissect the impact of each enzyme *in vivo*, we analysed the degree of NCAM polysialylation in eight mutant mouse strains differing only in the number of ST8SiaII and ST8SiaIV alleles. In brain of newborn mice, the polysialylation status of the expressed NCAM isoforms was determined by immunoblotting using anti-polySia and anti-NCAM antibodies. Polysialyltransferase mRNA levels were monitored by real-time RT-PCR.
Results. While total NCAM levels were similar in all genotypes, we observed gene dose-dependent alterations in the amount of polySia and in the ratio of polysialylated to non-polysialylated NCAM. In wild-type and ST8SiaIV-null mice, almost 100% of the expressed NCAM was in the polysialylated form, while the fraction of polySia-NCAM dropped to 55% and 30% in ST8SiaII-null and ST8SiaII-/-ST8SiaIV+/- mice, respectively. By contrast, a single functional allele of ST8SiaII (ST8SiaII+/- ST8SiaIV-/-) was sufficient to polysialylate >90% of the NCAM pool. In wild-type brain, ST8SiaII and ST8SiaIV mRNA level differed only by factor 2 and loss of one enzyme was not compensated by increased mRNA level of the second enzyme.
Conclusions. *In vivo*, the two polysialyltransferases show a remarkable imbalance in their capacity to polysialylate the whole NCAM pool. In the perinatal phase, loss of ST8SiaII resulted in premature expression of a large fraction of non-polysialyated NCAM

P169
DIFFERENTIAL EFFECTS OF C8 AND C16-CYCLOPROPENYLCERAMIDES ON CERAMIDE DESATURASE AND SERINE PALMITOYL TRANSFERASE ACTIVITIES

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The introduction of a 4,5-cyclopropene ring in the sphingoid base of C8 and C16 ceramides produced compounds showing inhibitory activity on several enzymes of sphingolipid biosynthesis when incubated with human lymphoblasts. However, depending on the constitutive fatty acid, the biological effects of the two ceramides were found to be different. Whereas an incubation of 24 h with either one of the two ceramides at 5 µM strongly blocked dihydroceramide desaturase activity, yielding mostly dihydroceramides, only C8-cyclopropenylceramide (GT11) inhibited by 70% the biosynthesis of ceramides using radioactive serine as a precursor, while C16-cyclopropenylceramide somewhat stimulated the synthesis of sphingolipids. When [^{3}H]-dihydrosphingosine was used as a precursor, the presence of GT11 in the incubation medium had no influence on the

labeling of sphingolipids, suggesting that the relevant target of GT11 concerning the inhibition of ceramide biosynthesis was serine palmitoyltransferase (SPT). However, the assay of SPT using purified microsomes showed an inhibitory effect when the microsomes were isolated from lymphoblasts preincubated with GT11, but no effect was seen when GT11 was added to the microsomes of control cells at the time of assay, thus ruling out any direct competitive inhibition of GT11. Moreover, the presence of the constitutive C8 fatty acid induced a time and dose-dependent cell death by triggering apoptosis when lymphoblasts were incubated with GT11. No such cytotoxic effect was seen with C16-cyclopropenylceramide at any concentration.

P170
CHANGES IN GLYCOSIDASE LEVELS IN FOUR CANCEROUS BREAST CELL LINES AS COMPARED TO A 'NORMAL' BREAST CELL LINE

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Introduction:
Breast cancer with an annual incidence of 36 000 is the most common cause of cancer related death among women in the UK and Wales. The evidence to-date suggests that posttranslational cellular glycoproteins levels are altered in breast cancer. However, the role of glycosidases in breast cancer formation and progression still remains an under-researched area of glycobiology.
Methods:
The activity of 10 glycosidases were assayed using a microplate colorimetric assay in three metastatic (namely;MDA MB 435, ZR 75-1 and MCF 7), one primary (BT474) and one normal (HB4a) human breast cell line. A detailed study of β-N-acetylglucosaminidase activity under a variety of conditions was performed for all five breast cell lines.
Results:
Significant changes ($P<0.05$) were observed in the activity of β-N-acetylglucosaminidase, β-mannosidase, β-N-acetylgalactosaminidase and α-fucosidase in cancer as compared to normal breast cell lines. However, no significant ($P<0.05$) difference was observed in the optimum pH and glycosidases activity under a range of different temperatures (4 - 65°C).
The kinetic analysis showed a statistically significant ($P<0.05$) increased β-N-acetylglucosaminidase Vmax level in the metastatic breast cell lines compared to primary and normal breast cancer cell lines. The Km remained unaltered.
Conclusion:
This is the very first time that increased glycosidase activities have been observed in the above cancerous breast cell lines and the increased β-N-acetylglucosaminidase Vmax level could correspond to an increased enzyme protein level in the tumour cells.

P171
FUCOSYLATION IN WORMS AND INSECTS

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Fucose is a key sugar in molecular interactions and it appears that the most complex N-glycans of invertrebrates contain this residue. For instance, *Caenorhabditis elegans* has a range of oligosaccharides containing between one and four fucose residues, whereas a major proportion of the N-glycans of *Drosophila melanogaster* adults contain core α1,6-linked fucose in addition to a small proportion carrying both core α1,3- and core α1,6-fucose. It has been shown previously that the modification of the N-glycan core by α1,3-fucose constitutes an epitope recognised by antibodies raised against horseradish peroxidase (anti-HRP). The modification is also known to be immuno- and allergenic. We have new evidence to indicate that structures carrying core α1,3-fucose are the source of the cross-reaction between anti-HRP and (probably neural) glycoproteins from both the fly and the worm. Recombinant forms of four *Drosophila melanogaster* core α1,3-fucosyltransferase homologues were expressed in *Pichia pastoris*, *Drosophila* Schneider 2 cells and Sf9 cells and have been tested for their capability to modify reducing terminal *N*-acetylglucosamine and to recreate the anti-HRP epitope. Anti-HRP binding analysis was also performed on various fly mutants and these data should aid further determination of the genetic basis of this modification. Also, recombinant forms of *Apis mellifera* core α1,3-fucosyltransferase homologues were expressed in *Pichia pastoris* and Sf9 cells and were tested for their capability to transfer a fucose residue to either reducing end *N*-acetylglucosamine or non-reducing terminal LacdiNAc. The latter data allow us to determine the candidate genes encoding the activities creating the allergenic core α1,3-fucose structure or the Lewis-like glycan, which is the most complex structure found in honeybee venom glycoproteins.
Modification of N-glycans in insects following addition of *N*-acetylglucosamine(s) to the non-reducing terminal of the N-glycan usually involves addition of one or two fucose residues to the core *N*-acetylglucosamine and subsequent removal of short arm N-acetylglucosamine by a hexosaminidase.
Furthermore, it was indicated that the modification constitutes an epitope recognised by antibodies raised against horseradish peroxidase (anti-HRP); the anti-HRP predominantly stains neuronal tissue of different insects (e.g. fruitfly, grasshopper) and 10% of *Caenorhabditis elegans* neurons.
end *N*-acetylgalactosamines. with the aim of identifying enzymes needed to create allergenic N-glycan structures as

well as the most complex N-glycan structure found in honeybee venom.

P172
EXPRESSION AND FUNCTIONAL ANALYSIS OF THE CMP-NEU5AC-SYNTHETASE FROM EMBRYONIC DROSOPHILA MELANOGASTER

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Sialylation is an important modification of glycoconjugates in the deuterostome lineage of animals. Dynamic addition and/or subtraction of sialic acids on preexisting glycotopes is implicated in many cellular mechanisms such as cell-cell interactions and host-pathogen recognition. Sialylation patterns have been demonstrated to show tissue and differentiation specific modulation and to vary between species (1). So far, sialylation has been detected in bacteria, protozoa, virus and fungi (2).
In Drosophila sialic acids were demonstrated throughout development by lectin staining and gas-liquid-chromatography-mass spectrometry (GLC-MS). Moreover, antibody-staining revealed the expression of a homopolymer of α2,8-linked sialic acid, polysialic acid, in 14- to 18-hour embryos (3). Two genes essentially involved in the sialylation pathway have recently been functionally characterized in Drosophila melanogaster. These are the Neu5Ac-9-P-synthase, which converts ManNAc-6-P into Neu5Ac-9-P (4) and an α2-6-sialyltransferase (5). In silico analyses of Drosophila gene libraries identified further genes, which may be involved in a potential sialylation pathway.
Here we report the cloning of the Drosophila homologue of the CMP-sialic acid synthetase using a PCR-based strategy. Primers used to clone the enzyme were based on two highly conserved motifs, which are present in all known CMP-Neu5Ac-synthetases. Studies describing the expression and the functional analysis of the newly identified gene in vivo and in vitro will be presented.

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P173
FUNCTIONAL ANALYSIS OF A LECTIN-LIKE DOMAIN OF UDP-GALNAC: POLYPEPTIDE N-ACETYL- GALACTOSAMINYLTRANSFERASE 1 (GALNAC-T1)

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Biosynthesis of mucin-type carbohydrates begins by transfer of GalNAc to Ser or Thr residues on acceptor proteins. This reaction is catalyzed by polypeptide GalNAc-transferases, which are characterized by a C-terminal lectin-like domain. We have shown that the lectin-like domain is involved in the GalNAc-T1 activity. To elucidate the catalytic role of the lectin domain, its sugar binding activity was investigated. We generated several lectin domain mutants of GalNAc-T1 using site-directed mutagenesis, and the binding activity of the wild type and the mutant enzymes to resins coated with various carbohydrates was examined. The wild type enzyme bound to the resins coated with GalNAc residues most efficiently, and this binding was inhibited by 100mM GalNAc. The lectin domain mutant, D444A/D484A, showed significantly weaker binding activity than the wild type. Moreover, the mutant deleted with the lectin domain lost the carbohydrate binding activity. These results indicated that the lectin domain of GalNAc-T1 directly bound to GalNAc residues, and that Asp444 and Asp484 are important residues for sugar binding. The recognition of GalNAc residues on the accepter peptides by the lectin domain may be involved in the follow-up activity. Interestingly, UDP inhibited the lectin activity of GalNAc-T1. This suggests that the enzyme releases the GalNAc-peptides upon binding with UDP-GalNAc. The released acceptor peptides, then, might bind to the catalytic domain of the enzyme, resulting in further glycosylation of the peptides. A binding study of GalNAc-T1 to GalNAc-peptides is in progress.

P174
POLYOXOMETALATES AS INHIBITORS FOR SULFOTRANSFERASES

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Sulfated glycans are involved not only in a variety of biological events including cell-cell interaction, but also in cancer progression and other diseases to be overcome. An approach to exploit medicines for the diseases would be finding of sulfotransferase (SulT)-specific inhibitors, but effective compounds have never been identified.
Polyoxometalates (PM) are inorganic polymeric anions and cluster molecules made of condensed MO_6 octahedra (M means metal atoms). Large numbers of PMs have been identified and some of PMs have anti-virus and anti-tumor activities in relation to their compositions and structures.

However, molecular mechanisms for explaining these activities still remain unclear.
Here we report that some kinds of PMs can inhibit SulT activities. We prepared crude membrane fractions of COS-7 cells expressing each sulfo- or glycotransferases and assayed the enzymatic activities in the presence of PMs. In the case of Gal 3-*O*-SulT-2 (Gal3ST-2) which is responsible for sulfation of various glycoproteins, vanadium-containing PMs had strong inhibitory effects and half-inhibitory concentration of the best one was 3 nM. Other tungsten-containing PMs were effective for cytosolic SulTs, and the dissociation constant between SULT1A3 and the best PM was 8.5×10^{-8} M by the surface-plasmon resonance method. These results suggested that some PMs, new type of sulfotransferase inhibitors, would serve as medicines and useful reagents for studies of sulfated glycans.

P175
A DHHC PROTEIN DEPENDENT GALNAC-TRANSFERASE INVOLVED IN DROSOPHILA GLYCOLIPID BIOSYNTHESIS.

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A common motif in N- and O-glycans of glycoproteins as well as in glycolipids is the disaccharide sequence Galβ1,4GlcNAc (lactosamine or lacNAc). This structure can be formed by a number of Golgi UDPGal:GlcNAcβ-R β1,4Galactosyltransferases that act on terminal GlcNAc residues. In a number of instances the lacNAc motif is replaced by GalNAcβ4GlcNAc (N, N'-diacetyllactosediamine or lacdiNAc). In mammals, lacdiNAc is restricted to specific proteins and tissues, while the lacdiNAc sequence seems to be more widespread in invertebrate glycoconjugates. In parasitic worms like Schistosoma mansoni lacdiNAc provides an antigenic determinant. The GalNAc-transferase responsible for the biosynthesis of the lacdiNAc structure has been cloned from different species.
We have identified a unique drosophila β4GalNAc-transferase that requires a second protein for the active expression in Chinese hamster ovary cells. This membrane protein is a member of a gene family with a conserved DHHC zinc finger domain. Initial experiments have shown that the two proteins are involved in the biosynthesis of glycosphingolipids. In drosophila, the lacdiNAc structure on glycolipids contains a phosphoethanolamine group β1,6-linked to GlcNAc (GalNAcβ1,4(PEtnβ1,6)GlcNAc). We are currently investigating the hypothesis if the DHHC protein is involved in the transfer of PEtn to the GlcNAc residue.

P176
MOLECULAR CLONIG OF A BETA-GALACTOSIDE ALPHA 2,3-SIALYLTRANSFERASE GENE OF *VIBRIO* SP. JT-FAJ-16 AND ITS EXPRESSION IN *ESCHERICHIA COLI*

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Sialyl-oligosaccharides are and will be of great importance in industrial applications as well as research purposes. The advantages of enzymatic sialylation over chemical sialylation include high reaction yields and stereoselectivity. Furthermore, bacterial sialyltransferases may be prepared easily in large quantities and very much stable compared to the mammalian enzymes. Therefore, we have been screening a large number of bacteria for novel sialyltransferase activities. During the course of the study, we isolated a marine bacterium which expressed an alpha 2,3-sialyltransferase activity. This bacterium appeared to be closely related to *Vibrio rumoiensis*, and was designated as *Vibrio* sp. JT-FAJ-16. Using the alpha 2,3-sialyltransferase gene from *Photobacterium phosphoreum* JT-ISH-467 as a probe, the genomic library prepared from JT-FAJ-16 was screened, and a gene encoded for a protein of 402 amino acids was identified. This protein showed homology of 64.7%, 30.5% and 27.3% to the alpha 2,3-sialyltransferase from JT-ISH-467, the alpha 2,6-sialyltransferase from *P. damselae* JT-0160 and the alpha 2,3/2,8-sialyltransferase from *Pasteurella multocida* subsp. *multocida* strain Pm70, respectively. The DNA fragments that encoded for the full-length protein and its putative mature form were amplified by PCR and cloned into expression vector pTrc99A. Both of the genes were expressed well in *E. coli*. It was revealed that total soluble proteins from both of the strains of *E. coli* showed a sialyltransferase activity, which transferred NeuAc from CMP-^{14}C-NeuAc to lactose. The alpha 2,3-sialylation of the PA-labeled lactose by the proteins was confirmed by HPLC analysis. This is the first report of a sialyltransferase from genus *Vibrio*.

P177
CLONING AND EXPRESSION OF HUMAN SIALIDASE NEU4 IN E. COLI

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Sialidases or neuraminidases are glycohydrolytic enzymes removing sialic acid residues from glycoproteins and

glycolipids. They are widely distributed amongst all living organisms, from microorganisms to vertebrates. In mammals, several sialidases with different subcellular localisations and biochemical features have been described: a lysosomal form, a membrane-associated form and a soluble enzyme. NEU4, the fourth member of the human sialidase family, has recently been identified. It consists of a short and a long form of 484 and 496 residues, which are associated to intracellular membrane structures or mitochondria, respectively. Interestingly, the expression of the long form of NEU4 in fibroblasts from patients affected by sialidosis or galactosialidosis results in clearance of lysosome storage material. These results suggest a possible role for NEU4 as "rescue enzyme".

In order to carry out a thorough kinetic and structural characterization of this enzyme, we cloned it in E. coli using pET102/D-TOPO as a vector. The protein was expressed in E. coli BL21 strain as a fusion protein with thioredoxin, and purified through affinity chromatography on a Ni-NTA resin (a histidine patch is inserted inside thioredoxin coding sequence).

After purification, an overnight incubation of the fusion protein with enterokinase allowed the release of NEU4 from its fusion partner.

A preliminary characterization shows that the enzyme is soluble and correctly folded; moreover the amount obtained (about 2 mg/l) should allow crystallization studies to be performed. Kinetic characterization of recombinant NEU4 is currently underway in our laboratory.

P178

EXPRESSION AND FUNCTIONAL CHARACTERIZATION OF BRAIN-SPECIFIC UDP-GALNAC: POLYPEPTIDE N-ACETYLGALACTOSAMINYLTRANSFERASES

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Mucin-type O-glycosylation is one of the most important post-translational modifications of proteins, and a UDP-GalNAc: polypeptide N-acetylgalactosaminyltransferase (GalNAc-transferase) catalyzes the initial step in biosynthesis of mucin-type glycans by transferring GalNAc from UDP-GalNAc to a peptide acceptor. This enzyme is biochemically important because it determines the number and positions of O-glycans in a protein. Recent studies on the molecular cloning of GalNAc-transferases revealed a large gene family, with 15 isozyme genes cloned to date. The large number of isozymes in the family suggests that O-glycosylation in the cell is regulated through distinctive sets of isozymes expressed in each tissue. Presence of tissue-specifically expressed isozymes supports this idea.

We previously cloned two brain-specific GalNAc-transferases, GalNAc-T9 and a putative GalNAc-transferase (pt-GalNAc-T). To characterize these isozymes biochemically, we first assayed pt-GalNAc-T for enzymatic activity using several typical mucin-like peptides as an acceptor substrate. pt-GalNAc-T was capable of glycosylating some of the peptides, with the highest activity toward a MUC5AC peptide (GTTPSPVPTT). This indicates that pt-GalNAc-T is a novel member of the GalNAc-transferase family, and this enzyme is, therefore, designated GalNAc-T16. We then compared expression pattern of GalNAc-T16 with that of GalNAc-T9 in the rat brain by *in situ* hybridization. Interestingly, GalNAc-T9 and -T16 were similarly expressed in the brain; they were most abundant in neurons of the hippocampus and cerebellum. Coexpression of these isozymes suggests that they might cooperatively glycosylate acceptor peptides in some neurons. We are currently attempting to identify neural cell types expressing GalNAc-T9 and -T16.

P179

STRUCTURAL AND ENZYMATIC CHARACTERIZATION OF RECOMBINANT GDP-D-MANNOSE DEHYDRATASE (GMD) FROM PBCV-1 VIRUS.

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GDP-D-mannose dehydratase (GMD) is a key enzyme for the biosynthesis of GDP-L-fucose, which is present from bacteria to human cells. We have demonstrated that a functional GMD is encoded also in *Paramecium bursaria Clorella* virus (PBCV-1) genome. PBCV-1 is a large polyhedral virus that replicate in unicellular Chlorella-like green algae and, unlike other viruses that use host-encoded enzymes in ER-Golgi system for glycoprotein production, PBCV-1 encodes many enzymes required to glycosylate its capsid proteins. Together with the dehydratase activity, GMD from PBCV-1 displays also an unusual NADPH-dependent reductase activity, which leads to GDP-D-rhamnose formation.

We have produced recombinant PBCV-1 GMD with the aim to further analyze the structural and enzymatic properties of this enzyme and to compare it with GMD from prokaryote and eukaryote sources. Viral GMD resulted a homodimer. The recombinant protein contained tightly bound NADPH, with a ratio of one dinucleotide molecule per monomer. Unlike other nucleotide-sugar dehydratase, NADPH, not $NADP^+$, seems to be the active cofactor for both dehydration and reduction. Moreover, NADPH, together with the catalytic role, played an

important structural role, since after its oxidation to $NADP^+$, it was released from GMD and the protein dimer was dissociated to an inactive monomeric form.
Comparison of PBCV-1 properties with that obtained for recombinant *E. coli* and human GMDs indicated that the viral enzyme shares some properties with *E. coli* GMD. Conversely, the behaviour was significantly different from that observed for the human enzyme, suggesting different roles and regulatory properties between viral, prokaryotic and eukaryotic GMDs.

P180
EFFECTS OF NEU2 SIALIDASE OVER-EXPRESSION IN MYELOID LEUKEMIC CELLS, K562

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Abnormal sialylation is a key event in oncogenic transformation and is related to malignant properties such metastasis. Cellular sialic acid levels are modulated mainly by sialidases; remarkable alterations of endogenous sialidases expression, revealed in cancer, suggested an intriguing involvement in the disease.
The cytosolic enzyme Neu2 is characterized by a broad substrate specificity and seems to be related to the metastatic potential in highly invasive cell lines.
In this study, we over-expressed Neu2 in chronic myeloid leukemic cells, K562 and had evidence that cytosolic sialidase is able to interfere with the neoplastic properties, modulating sialo-glycoconjugates.
K562 cells were transfected with pcDNA-NEU2 and stable transfectants were selected by geneticin. Cell sphingolipids were analyzed by TLC and radiochromatoscanner after metabolic labelling with [^{3}H]sphingosine while sialo-glycoproteins were identified with DIG Glycan Differentiation kit.
Proliferation rate was assayed by [^{3}H] thymidine incorporation and soft agar assay; apoptosis sensitivity was revealed analysing caspase activation by Western Blot after serum deprivation.
NEU2 expression in K562 cells gave rise to an enzymatic activity detectable in the soluble fraction after cell lysate fractionation. Neu2 action altered cellular gangliosides determining a marked decrease of GD1a (-42%). Moreover, the glycoprotein profile was deeply affected with a consistent decrease of sialylation of some cytosolic proteins (66-55KDa). These modifications influence interestingly the tumoral features slowing down proliferation rate and increasing apoptosis susceptibility. Further experiments are in progress in order to better elucidate Neu2 action in cancer cells and identify interferences Neu2 mediated on the signal transduction cascade.

P181
PURIFICATION, CLONING, AND EXPRESSION OF AN ALPHA 2,3-SIALYLTRANSFERASE FROM *PHOTOBACTERIUM PHOSPHOREUM* JT-ISH-467

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The oligosaccharide chains in glycoconjugates, such as glycoproteins and glycolipids, are playing important roles in interaction between cells, cell differentiation, cell multiplication, *etc*, in diverse organisms. Bacterial glycosyltransferases have been shown to be convenient tools for syntheses of various oligosaccharides, which are key materials for the functional studies, because, in general, bacterial enzymes are easier to prepare in large quantities and stabler than eukaryotic counterparts. Thus we have been searching for bacteria with novel glycosyltransferase activities. A novel alpha 2,3-sialyltrasferase was isolated in the process from the cell lysate of a luminous squid habitant, *Photobacterium phosphoreum* JT-ISH-467. The enzyme catalyzed transfer of NeuAc from CMP-NeuAc to galactosides of lactose and lactosamine. The purified enzyme had a molecular mass of 39 kDa in SDS-PAGE analysis. The gene encoding for the alpha 2,3-sialyltrasferase was cloned from the genomic library of the bacteria using probes derived from the N-terminal and internal amino acid sequences. The nucleotide sequence was then determined, and an open reading frame (1,230 bp) for a 409 residue protein with a predicted molecular mass of 46.7 kDa was identified. The amino acid sequence of the protein shows 32% homology to the alpha 2,6-sialyltrasferase in *P. damselae*. Since the purified enzyme lacked the 21 amino acids in the N-terminus, the molecular mass of the mature protein was calculated to be 44.3kDa, which was close to the measured value. The gene was inserted into expression vector pTrc99A, and it was demonstrated that the protein expressed in *Escherichia coli* had retained the activity.

P182
DIFFERENTIATION OF MOUSE NEUROBLASTOMA CELLS INDUCED BY SHORT HAIRPIN RNA TRANSFECTION OF PLASMA MEMBRANE SIALIDASE NEU3.

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Several evidences suggest that gangliosides play an essential role in modulating functions of neuronal cells. Ganglioside patterns in the nervous system dramatically change during development, neurite outgrowth, synaptogenesis and malignant transformation. Sphingolipid biosynthesis is necessary for neuritogenesis in primary cultures of hippocampus neurons and in neuroblastoma cells. In this work we present data on the possible role of plasma membrane associated sialidase Neu3 in determining the cell surface ganglioside composition (i.e. the cell surface organization) and how this could be related to the process of cell differentiation.
We silenced the Neu3 gene in neuroblastoma cells using: lentivirus vector containing shRNA for membrane associated sialidase Neu3 gene and selecting stable clones, or lentivirus vector coding for tetracycline-inducible expression system and then infecting with inducible lentivirus vector containing shRNA for Neu3.
Results showed that down-regulation of gene caused morphological changes, mainly neurite outgrowth, and a strong reduction in the growth of cells. We also observed a reduction in the cell contacts. The sphingolipids pattern was dramatically different in Neu3 knockdown cells. We suggest that Neu3 gene plays an important role in controlling the differentiation and cell contacts.

P183
SPECIFICITY OF THE BOVINE SIALATE 7(9) *O*-ACETYLTRANSFERASE

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The sialic acid family consists of about 50 members including *O*-acetylated species. These are found in various bacterial species and in animals from the echinoderms to mammals. They are involved in cellular and molecular recognition, growth and morphogenesis, immune activation, apoptosis, tumor growth, and they are bacterial virulence factors and receptors for some viruses. Sialic acid *O*-acetylation is carried out by AcCoA:sialate 7(9)-*O*-acetyltransferase and a corresponding 4-*O*-acetyltransferase, which are localised in Golgi membranes (1,2).
The sialate 7(9) *O*-acetyltransferase is being studied in the microsomes from bovine submandibular glands. The assay system uses radiolabeled AcCoA to determine the enzyme activity by chromatographic methods. Membrane-bound and detergent-solubilised forms of the enzyme show different activity, regarding time, pH, temperature and protein concentration. The enzyme shows highest activity with CMP-Neu5Ac and free Neu5Ac, whereas de-*O*-acetylated bovine submandibular gland mucin is only a weak substrate. Gangliosides and sialyllactose are inactive. The addition of dialysate from solubilised microsomes to the assay mixture enhances enzyme activity, indicating the presence of a low molecular compound as a possible activator of the enzyme.

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P184
IDENTIFICATION OF INTERACTING PROTEINS OF THE UDP-GLCNAC 2-EPIMERASE/MANNAC KINASE BY YEAST TWO HYBRID SCREENING

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The UDP-GlcNAc 2-epimerase/ManNAc kinase is the key enzyme in the de novo synthesis pathway of sialic acids and a regulator of cell surface sialylation (Keppler et al., 1999, Science 284, 1372). It is a bifunctional enzyme catalyzing the conversion of UDP-*N*-acetylglucosamine to *N*-acetylmannosamine and the phosphorylation to *N*-acetylmannosamine 6-phosphate. The polypeptide of this enzyme self-associates as a dimer and as a hexamer. The hexamer is fully active in both enzyme activities, whereas the dimer possesses only the kinase activity (Hinderlich et al., 1997, J Biol Chem 272, 24313). In the final step of the biosynthetic pathway *N*-acetylneuraminic acid (NeuAc) is activated by the addition of CMP. Remarkably the activation of the NeuAc occurs in the nucleus in contrast to other amino sugars activated in the cytosol. It has been shown that the enzyme activity is increased if protein kinase C (PKC) phosphorylates the enzyme (Horstkorte et al., 2000, FEBS Lett, 470 (3), 315). During times of intense plasticity within the nervous system, sialylation differs from times of maintenance. In order to understand the underlying regulatory mechanisms, we tried to idenify UDP-GlcNAc 2-epimerase/ManNAc kinase-interacting proteins. Using a human fetal brain cDNA library we identified 4 proteins interacting with the UDP-GlcNAc 2-epimerase/ManNAc kinase in a Yeast Two Hybrid Screen.

P185
FUNCTIONAL EXPRESION OF ENZYMES INVOLVED IN THE SALVAGE PATHWAY OF L-FUCOSE

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L-fucose is an important monosaccharide bound to terminal parts of cell surface glycoconjugates in humans and other mammals. Fucosylated glycans play an important role in several biological processes and in pathological events. They initiate extravasation of leukocytes in inflammation and regulate selectin-mediated leukocyte endothelial adhesion, host-microbe interaction, fertilization, development, tumor metastasis and apoptosis. Aberrant fucosylation is furthermore associated with pathophysiology of the leucocyte adhesion deficiency syndrome.
In mammals there exist two different cytosolic pathways for formation of the activated form of L-fucose, the GDP-fucose. The *de novo* pathway is based on the conversion of GDP-mannose to GDP-fucose by the FX enzyme. The salvage pathway utilizes fucose from extracellular sources or from intracellular degradation of glycoconjugates. First, fucose 1-phosphate is formed by the action of L-fucose kinase. GDP-fucose pyrophosphorylase then condenses GTP and fucose 1-phosphate to GDP-fucose, which is finally transported to the Golgi apparatus, where it serves as a substrate of fucosyl-transferases in glycoconjugate biosynthesis.
Here we report the recombinant expression of murine and human enzymes of the fucose salvage pathway. Full lenght L-fucose kinase and GDP-fucose pyrophosphorylase were produced in insect cells by the baculovirus system, whereas separate functional domains of the enzymes were expressed in E.coli. We will present biochemical features of all enzymes investigated.

P186
SUBSTRATE SPECIFICITY STUDY OF α1,3-FUCOSYLTRANSFERASE FROM *HELICOBACTER PYLORI*

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As a major gastroduodenal pathogen, *Helicobacter pylori* is well known for the major cause of gastric and duodenal ulcer. The infection is also associated with atrophic gastritis, a precondition to gastric cancer and lymphoma. *H. pylori* can express fucose-containing Lewis antigens as a molecular mimicry to the cell surface glycoconjugates of gastric epithelial cells to avoid the detection of host immune system. Such mimicry also plays an important role in mucosal adhesion, immune evasion and auto-antibody induction. Fucosyltransferases (FucTs) are the enzymes responsible for the last steps in the biosynthesis of Lewis carbonhydrate determinants, including Le^a, Le^b, Le^x, Le^y, sLe^x and sLe^y. The substrate specificity of α1,3-fucosyltransferase is of interest because it is reported that this enzyme has distinct Type ¯or Typeα substrate specificities and different ability for terminal or internal fucosyltion. We have cloned the FucT from *H. pylori* NCTC11639 and expressed in *E. coli* for further studies. Wild type FucT was expressed at a high level but in a insoluble form. Several C-terminal truncations were constructed to enhance the protein solubility and activity. The result of our studies will address (1) if different truncated forms have altered specificity, (2) the order of fucosylation in long chain oligosaccharides.

P187
CEREBROSIDE SULFOTRANSFERASE IS A HOMODIMER WHOSE GOLGI LOCALIZATION IS DETERMINED BY ITS TRANSMEMBRANE DOMAIN

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Cerebroside sulfotransferase (CST) is a Golgi resident enzyme catalyzing the final step in the synthesis of several sulfated glycolipids, like sulfatide, seminolipid, and sulfated lactosyl ceramide. Sulfatide and seminolipid play essential roles in myelin formation and spermatogenesis, respectively. Using immunofluorescence studies with epitope-tagged CST variants we obtained evidence for localization of CST in the medial/trans Golgi. Immunoprecipitaion experiments revealed oligomerization of CST, which occurred in the endoplasmic reticulum. Oligomerization is mediated by the lumenal domains of the enzyme. Glycerol density gradient centrifugation indicated that the native CST protein is a homodimer. Carboxy- and amino-terminal deletion mutants fused to the green fluorescent protein (GFP) were generated in order to obtain information about the signals required for Golgi localization of CST. Our data show that the first 36 amino acids of CST are sufficient to localize GFP to the Golgi apparatus. Moreover, deleting the cytosolic tail except for the membrane domain flanking lysine residues did not affect its subcellular localization. Thus, the transmembrane region of CST contains sufficient information for Golgi localization. Replacing hydrophilic amino acids in the transmembrane domain of CST by leucine residues did not affect Golgi localization. Together these data suggest that the transmembrane domain of CST is sufficient for Golgi localization of CST. However, a specific sequence motif mediating Golgi localization could not be identified.

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HIGH EFFICIENCY PRODUCTION AND ENZYMATIC CHARACTERIZATION OF A SOLUBLE RECOMBINANT DERIVATIVE OF A MARINE BACTERIAL ALPHA 2,6-SIALYLTRANSFERASE

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Beta-galactoside alpha 2,6-sialyltransferase from *Photobacterium damselae* JT0160 shows unique acceptor specificity. For example, this enzyme catalyzes the transfer of NeuAc to fucosyl- and sialyl-trisaccharides. The deduced amino acid sequence of the enzyme appears to have a multi-domain structure; the N-terminal hydrophobic domain with a signal function, the putative catalytic domain and the C-terminal domain with a putative membrane-binding function. Thus this enzyme seems to be quite different in structure from those of mammalian sialyltransferases. We constructed a number of expression vectors, which contained a series of truncated sequences of the sialyltransferase and proteins were produced in *Escherichia coli* to identify portions of the protein essential for the activity. It was confirmed that the central domain had the catalytic function. Interestingly, active gene products were quite different in the amount of proteins produced in *E. coli*. One of the truncated proteins designated as N2C1, which consisted of 393 residues corresponding to 58% of the full length protein N0C0, was expressed about 30 times higher than N0C0. N2C1 protein was found in the soluble fraction from the lysate of the E. coli. Because N2C1 lacked the C-terminal domain, the hypothesized membrane-binding role of the domain was supported by this observation. We were then able to purify N2C1 enzyme 130-fold from the lysate and the enzyme migrated as a single band on SDS-PAGE. The Km value for CMP-NeuAc of N2C1 was 8μM, which is lower than the value, 15μM, of the native enzyme prepared from *P. damselae* JT0160.

P189
MEMBRANE-ASSOCIATED SIALIDASE NEU3 SHOWS AN EXTENSIVE INTRACELLULAR DISTRIBUTION AND LOCALIZES TO RECYCLING COMPARTMENT

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NEU3 is a member of the sialidase protein family known to be associated to the plasma membrane, with high enzymatic specificity toward gangliosides localized also on the cell surface of neighbouring cells. In order to better characterize its cellular localization, the *Mus musculus* NEU3 protein tagged with the HA-epitope at the C-terminus was transiently expressed in mammalian cells. By cell-surface protein biotinylation, nearly 20% of the protein was detected at the plasma membrane. The intracellular fraction of the protein largely colocalized with transferrin receptor positive structures, representing the recycling endosomes. This suggests that NEU3 may recycle between the plasma membrane and the recycling endosomal compartment. Moreover, subcellular fractionation based on discontinuous sucrose gradient reveals that the protein is present, as expected, in plasma membrane enriched fractions and noteworthy also in low density fractions known to contain endosomal and trans-Golgi network markers. Cell surface detection of NEU3 tagged either at the N-terminus or at the C-terminus with the HA-epitope indicates that both extremities face the extracellular environment. This is in agreement with the computational analysis of the protein sequence that does not reveal any transmembrane amino acid stretches. NEU3 could not be solubilized either by high salt concentrations or extreme pH treatments indicating that its membrane-anchoring does not occur by usual hydrophilic interactions.

Subcellular aspects of glycoconjugates metabolism

P190
A HUMAN GOLGI UDP-XYLOSE TRANSPORTER

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Most of the glycosylation reactions involved in the biosynthesis of glycoconjugates occur in the Golgi apparatus. They require nucleotide sugar donors that are synthesized in the cytoplasm before being transported and concentrated in the lumen of the Golgi compartment by specific transporters. The latter form a family of structurally conserved type III proteins named Solute Carrier 35 (SLC 35) and thus many putative nucleotide sugar transporters (NSTs) can be predicted from the existing databases.
Transport assays carried out *in vitro* with Golgi vesicles isolated from mammalian cells, showed specific uptake for a total of eight nucleotide sugars. Meanwhile, the proteins transporting these nucleotide sugars have been cloned with the exception of the UDP-xylose and UDP-glucose transporters. Aiming at identifying these elusive NSTs, 10 human open reading frames were selected by bioinformatics methods and heterologously expressed in yeast. Transport capabilities for UDP-Glc and UDP-Xyl were determined with Golgi vesicles

isolated from the resulting transformed cells. Vesicles from yeast cells expressing the human gene SLC35B4 showed specific uptake of UDP-Xyl and subsequent testing of other nucleotide sugars revealed a second activity for UDP-GlcNAc. Expression of the epitope-tagged SLC35B4 in mammalian cells, demonstrated strict Golgi localization. Because decarboxylation of UDP-GlcA is known to produce UDP-Xyl directly in the ER and Golgi lumen, our data demonstrate that two ways exist to deliver UDP-Xyl to the Golgi apparatus.

P191
ANALYSIS OF PUTATIVE GLYCOSIDASES FOR THE METABOLIC PATHWAYS OF CYTOSOLIC FREE N-LINKED GLYCAN SPECIES IN DROSOPHILA MELANOGASTER

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Recently it was revealed that misfolded proteins or others to be metabolized are excluded from the cells by ERAD (endoplasmic reticulum associated
Degradation) system. It has been suggested that the deglycosylation in cytosol, catalyzed by PNGase is important for facilitating the proteolysis of such glycoproteins.
It is considered that the free glycans released from glycoproteins by the action of PNGase or other ways are delivered into lysosome for complete digestion into monomeric sugars by the serial actions of lysosomal glycosidases. Some observations of partially degraded cytosolic free N-linked glycan moiety in the cytosol support the idea that such glycans are processed certain extent before being translocated into lysosome by the receptor which recognizes free glycans with specific structure. However, the details of this system are not elucidated yet.
Currently we are working on establishing some biological systems, such as cell cultures and some model organisms, to decipher the metabolic pathways of free N-linked glycans and their biological significance. Here we will present our work on Drosophila melanogaster. In the Drosophila genome project database, some of the candidate genes, which may be involved in the pathways were found: PNGase, ENGase, and six uncharacterized class II mannosidases with whose functions unknown. We are currently investigating the properties of those enzymes, such as intracellular localizations and enzymatic profiles. We also are analyzing the gene-knockdown phenotypes of each gene by inducible RNAi system.

P192
ANALYSIS OF THE FREE OLIGOSACCHARIDES FROM C. ELEGANS LACKING ENDO-&BETA;-N-ACETYLGLUCOS -AMINIDASE GENE

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PURPOSE. Although it is known that there are free oligosaccharides (FOS) in the cytosol of eukaryotic cells, their metabolic processes and physiological roles were unclear. Endo-β-*N*-acetylglucosaminidase (ENGase), which can cleave of *N*,*N'*-diacetylchitobiose moiety at their reducing ends *N*-glycans in glycoproteins, is assumed to be implicated in the FOS metabolism in the cytosol. To address them, we have analyzed the FOS from *C. elegans* wild-type (N2) and the mutant *eng-1(tm1208)* lacking ENGase.
METHODS. Extract of worm was dialyzed against deionized water and FOS was purified from the resulting outer solution. After fluorescence labeling with 2-aminopyridine at reducing ends of FOS, they were further purified by ConA-Sepharose affinity column chromatography. Structures of FOS were determined by comparison with standard oligosaccharides on HPLC, combination of HPLC and digestion with exo-, endo-glycosidases, and mass spectrometry.
RESULTS. High-mannose type oligosaccharides with one *N*-acetylglucosamine residue at their reducing ends (GN1) were found as major components of the FOS from wild-type. On the other hand, most FOS from *eng-1(tm1208)* had two *N*-acetylglucosamine residues at their reducing ends (GN2). This indicates that ENGase is involved in conversion of FOS-GN2 to FOS-GN1.

P193
STRUCTURAL ANALYSIS OF FREE N-GLYCANS OCCURRING IN WILD TYPE AND CYTOSOLIC α-MANNOSIDASE GENE DELETED C. ELEGANCE

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PURPOSE. In the cytosol of animal cells, free high-mannose type (HM) *N*-glycans have been found though the degradation pathway or the functional meaning of such oligosaccharides remain to be understood. In this report, for the elucidation of physiological role(s) of free HM *N*-glycans, we established a *Caenorhabditis elegans* mutant lacking the part of ORF F58H1.1, which is a putative gene for a cytosolic α-mannosidase, and analyzed the structural feature of free *N*-

glycans occurring in the both mutant and wild type worm (N2 Bristol).
METHOD. Worms were homogenized in Tris-HCl buffer, pH 8.5, and dialyzed. The resulting outer solution was desalted using gel filtration and ion exchange chromatography. After pyridylamination, the resulting fluorescence-labeled *N*-glycans were purified by Con A affinity chromatography, reverse-phase and size-fractionation HPLC. The structures were analyzed by a combination of α-mannosidase digestion and ESI-MS.
CONCLUSION. From wild type *C. elegans*, Man9-5GlcNAc1 were found at micromolar level. The ration of each glycan was as follows: Man5:Man6:Man7:Man8:Man9 = 12:9:9:6:1. The result of α-1,2 mannosidase digestion indicated that these free *N*-glycans have a common structural unit, Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAc. These structural features of free HM *N*-glycans in *C. elegans* were very similar to those from plant cells rather than animal cells. Interestingly, main structures of free *N*-glycans found in the mutant worm were Man5GlcNAc1 and Man6GlcNAc1, although expression of a cytosolic α-mannosidase gene was suppressed, suggesting that there may be an alternative pathway for free *N*-glycan degradation.

P194
GLUCOSIDASE II RECOGNIZE YHE HIGH-MANNOSE TYPE OLIGOSACCHARIDE MOIETY OF GLYCOPROTEINS

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Processing of the Asparagine (N)-linked oligosaccharides is processed by the removal of the terminal glucose residue by the glucosidase I and two inner glucose residues are then removed by the Glucosidase II (GII). Some of the mannose residues from Man9GlcNAc2 are trimmed by ER-mannosidases, and then the trimmed glycoproteins are transported to the Golgi apparatus. It has been proposed that N-linked oligosaccharides are important the quality control mechanism of the glycoproteins.
We investigated the detailed interaction of GII with glycoproteins containing oligomannosidic oligosaccharides by the use of the purified GII and prepared columns including ribonuclease B (RNase B), thyroglobulin (TG), and ovalbumin (OVA). Here, we show that the mannose-specific binding activities of GII to glycoproteins containing oligomannosidic saccharides requires both oligosaccharides and the protein moieties of the glycoproteins, and that two isoforms of GII, GII-H and –L (lower molecular weight isoform of GII), have different recognition patterns of glycoproteins. We examined the mannose-specific binding of GII. The GII-L and GII-H bind to various treated RNase B-columns by mannose-specific binding activities, but they don't bind to its glycopeptides and oligosaccharides columns. Furthermore, GII-H binds to variously treated TG columns by mannose-specific binding, but it doesn't bind to its glycopeptides and oligosaccharides columns. GII-L and –H strongly bind to variously treated RNase B- and Tg- columns, respectively.
GII recognizes the Man9GlcNAc2 on the proteins and Man8GlcNAc2 on the proteins. From these results, we speculated that GII participate in the quality control of the glycoproteins.

P195
CHANGES IN N-GLYCAN-METABOLISM DURING TOMATO FRUIT MATURATION: STRUCTURE AND PUTATIVE FUNCTION OF PLANT FREE *N*-GLYCANS.

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[Introduction] We postulate that free *N*-glycans occurring in developing plant cells might have a critical role in biological processes such as germination or fruit ripening. In this study, we analyzed changes in *N*-glycan-metabolism (changes of concentration, structures, and some enzyme activities) in association with tomato fruit ripening, as a part of study for elucidation of physiological function of the glycans. [Methods] Tomato fruits (KGM993) in each ripening stage (Mature green, Breaker, Pink, and Red) were homogenized in the Tris-HCl buffer, and the resulting homogenates were dialyzed against water. From outer solution, pyridylaminated (PA-) sugar chains were prepared and were purified by a Con-A affinity column, SF-, and RP-HPLC. The structures of the PA-sugar chains were determined by a combination of exo-glycosidase digestions and ESI-MS. Endoglycosidase and α−mannosidase were partially purified from each ripening stage tomatoes by HPI- and IEC-HPLC. Enzyme activities were assayed using high-mannose type PA-sugar chains as substrates. [Results] We revealed that the concentration of free *N*-glycans and endoglycosidase activity in the cytosol increased significantly in the period of pink-stage. Structural analysis of free *N*-glycans occurring in each maturing stage revealed that (1) in the green mature and breaker stages, the relative amount of Man9-5GlcNAc1 is almost the same, (2) in the pink and the red stages, the amount higher molecular species (Man9-8GlcNAc1) occur predominantly, suggesting that different species of α–mannosidase with different substrate specificities are expressed during the fruit ripening. Tomato endoglycosidase was highly active towards high-mannose type N-glycans with Manα1-2Manα 1-3Manβ1-unit.

P196
COMPARATIVE STUDIES OF 4-METHYLUMBELLIFERONE-DERIVATIVES-MEDIATED INHIBITORY EFFECT ON HYALURONAN SYNTHESIS

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Introduction: Hyaluronan (HA) is a ubiquitous, major component of the extracellular matrix and is crucial for various physiological processes. We have previously reported that 4-methylumbelliferone (MU) is an inhibitor of HA synthesis. We have also demonstrated that its inhibitory effect results in the suppression of matastasis of malignant melanoma cells. In this study, we performed comparative studies of the structures and inhibition of HA synthesis of MU-derivatives.
Materials and methods: Cell lines of human pancreatic cancer (KP1-NL) and the colon cancer (DLD-1) were treated with 100μM MU and its derivatives for 72 h, respectively. HA synthesis of each cell were quantified by an HA measurerment kit (Seikagaku Corporation), respectively.
Results: HA in the cells cultured with MU derivatives with a hydroxyl group and methyl group reduced the HA synthesis more than those without them. In addition, the inhibitory effect of HA synthesis by MU-derivatives having two hydroxyl groups is higher than that by those with one hydroxyl group.
Discussion: It is suggested that both the hydroxy group and methyl group of MU derivatives were involved in the inhibitory effect of HA synthesis. Specifically, the hydroxyl group strongly inhibited HA synthesis.

P197
FUNCTIONAL FEATURE AND GENE EXPRESSION OF PLANT ENDO-β-*N*-ACETYLGLUCOSAMINIDASE

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[Introduction] Free *N*-glycans occur in micromolar concentration in plant cells during their differentiation, growth and maturation stages. We assume that such free *N*-glycans may play a critical role for plant cell development or growth. In this study, as a part of study for elucidation of physiological function of such free *N*-glycans, we purified, characterized an endo-β-*N*-acetylglucosaminidase (endo-OS) from rice cells, and we determined partial amino acid sequences and expressed the gene in *E.coli*.
[Methods] Endo-OS has been purified to homogeneity from rice cells. Internal AA sequences were determined by ESI-MS using several tryptic peptides. Endo-OS gene was expressed using a cDNA clone (AK112067.1) corresponding all the sequences. Structural analysis of *N*-glycans was performed by a combination of 2D-sugar Mapping, ESI-MS and exoglycosidase digestion.
[Results and Discussions] Endo-OS having a molecular weight of about 70 kDa showed a strong activity toward the high mannose-type *N*-glycans with the Manα1-2Manα1-3Manβ1-unit as well as other plant endoglycosidases. Some internal AA sequences were in remarkable agreement with those deduced from a function unknown cDNA clone AK112067.1, indicating that it might encode the endoglycosidase. The cDNA clone product showed strong endoglycosidase activity with similar substrate specificity of endo-OS. Furthermore, we found high-mannose type free *N*-glycans bearing one GlcNAc occur predominantly in the cytosol fraction but almost all glycoproteins in the plant cells carry plant complex type *N*-glycans, suggesting that the endogenous substrate for endo-OS may be misfolded glycoproteins or glycopeptides produced in the ERAD system.

P198
HEPARANASE EXPRESSION IN BREAST CANCER PATIENTS

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Heparanase is an endo-β-glucuronidase that degrades heparan sulfate proteoglycan and seems to be involved in cancer development and metastasis. We had the objective to evaluate heparanase mRNA expression in mononuclear fraction of peripheral blood of 30 breast cancer patients compared with 20 healthy women by semiquantitative RT-PCR. Association between variables as heparanase expression, type of treatment, presence or absence of metastasis was assessed using univariate analysis by Students t-Test for parametric variables. Data management and analysis were done using the SPSS software, version 10 (SPSS Inc., IL, USA). Breast cancer patients expressed significantly high levels of heparanase, while healthy women no heparanase expression was observed. We obtained a correlation ($p = 0.04$), between low heparanase expression and patients submitted to tamoxifen treatment ($78.82 \Sigma 54.20$) compared with chemotherapy or radiotherapy treatment ($136.28 \Sigma 57.32$). It was also found a significant heparanase expression decreased after surgery ($p = 0.002$). In addition, an increased of heparanase expression was also obtained before metastasis ($81.01 \Sigma 17.01$) compared after metastasis ($142.90 \Sigma 59.71$) ($p = 0.027$). Seventy percent of lymphocytes of breast cancer patients were labeled with heparanase polyclonal antibody while only 10% of healthy

women limphocytes were labeled. However, when healthy women lymphocytes were incubated with plasma of breast cancer patients or with MCF-7 cells, they increased heparanase expression. Our data led us to conclude that heparanase can be a possible marker for breast cancer and also suggest an activation mechanism of lymphocytes' heparanase expression by tumor cells. Supported by FAPESP, CAPES, NEPAS

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IDENTIFICATION AND CHARACTERIZATION OF A NOVEL BIOMARKER GLYCOPROTEIN ASSOCIATED WITH AGING

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Introduction: Alzheimer's disease (AD) is thought to be pathological aging. Neurofibrillary tangle is one of the histopathological hallmarks of AD. It is composed of hyperphospholylated tau, microtube associated protein that normally form the cytoskeleton. We reported that tau prepared from the brain of AD patient is N-glycosylated. Aberrant glycoprotein synthesis may occur in AD brain because tau is a cytosolic protein. We recently found the increase of cytosolic N-glycosylated proteins during normal aging by comparative analysis with 2D-PAGE and Con A staining, and tried to identify and characterize these N-glycosylated proteins.
Experimental approaches: Cytosolic N-glycosylated proteins were concentrated by precipitation with Con A-agarose and separated by 2D-PAGE. Identification of cytosolic glycoproteins was performed by combination with tryptic digestion and nanoLC-ESI-Q-TOF/MS. The amounts of cathepsin D were determined by 1D-PAGE and immunoblotting with anti-rat cathepsin D antibody.
Results: Three cytosolic glycoproteins increased during aging were identified as cathepsin D, a lysosomal protease. Cytosolic cathepsin D had high mannose and/or hybrid type N-glycans because it was deglycosylated by Endo H. Cytosolic cathepsin D increased linearly during aging but it was not due to the lysosomal membrane disruption. This phenomenon was also observed in the hippocampus, cerebellum, kidney, liver and spleen.
Discussion: Cytosolic cathepsin D would be expected as a new biomarker of aging. It remains to be elucidated the reason why cathepsin D accumulated in the cytosol during aging.

P200
ANTITUMOR EFFECT OF CHEMICALLY SYNTHESIZED NOVEL GLYCOCONJUGATES

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Tumor-related expression of fucosylated antigens and its suppression by cell-mediated priming using sugar acceptors for fucosyltransferases were previously demonstrated in colorectal cancer cells (1). Treatment of these cells with GlcNAcβ1,3Galβcholestanol at low concentrations caused a significant suppression of sialylated Le^X and YB-2 (1) antigens on the cell surface, while at higher concentrations, the cytotoxicity of the sugar-cholestanol was also observed in a variety of cancer cells. In this study, newly synthesized glycoconjugates with cholestanol aglycons were evaluated for their antitumor actions both *in vitro* by a cell proliferation inhibition assay and *in vivo* in a mouse model of peritoneal dissemination. Liposomes encapsulated or cyclodextrin included sugar-cholestanols could be used for both *in vitro* and *in vivo* investigations even though sugar-cholestanols *per se* were insoluble in water. GlcNAcβ1,3Galβ cholestanol added to the cell medium was found to be taken up by the cells, and then glycosylated through intercellular glycosylation pathways. Then the glycosylated, primed sugar-cholestanol was secreted. By increasing the concentration of the sugar-cholestanol, the viability of the cells was found to decrease sharply together with apoptotic changes induced in a short period of time. Further, intraperitoneal treatment of liposomes encapsulated GlcNAcβ1,3Galβ cholestanol in the mouse model of peritoneal dissemination showed that tumor growth was suppressed and the survival rate was significantly improved. These observations led us to conclude that GlcNAcβ1,3Galβ cholestanol might be effective for the prevention and treatment of peritoneal metastasis. (1) S. Yazawa, *et al*., Glycobiology, 12:545-553, 2002.

Regulatory mechanisms in glycoconjugates metabolism

P201
DETERMINATION OF MAJOR NEUTRAL OLIGOSACCHARIDES IN HUMAN MILK IN EARLY LACTATION STAGE

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Human milk oligosaccharides are thought to act as prebiotics as well as soluble receptor anologues; they compete with pathogenic bacteria, bacterial toxins and viruses for attachment to gastrointestinal receptor sites. It is suggested that early lactation milk is most important to protect the infants against infection of pathogenic microorganisms. However, the concentrations of each human milk neutral oligosaccharide in early lactation have not been determined yet. The present study has been conducted to determine the contents of each neutral oligosaccharide at the start of lactation (Day1) and the two consecutive days (Day2 and 3), and also to observe the changes of oligosaccharides levels in this stage. The human milk oligosaccharides investigated here were 2'-fucosyllactose (2'-FL), 3-fucosyllactose (3-FL), lacto-N-difucotetraose (LNDFT), lacto-N-tetraose (LNT), lacto-N-neotetraose (LNnT), three lacto-N-fucopentaose (LNFP1, 2 and 3) and two lacto-N-difucohexaose (LNFDH1 and 2). The neutral oligosaccharide fractions with internal standard (isomaltotoriose and isomaltohexaose) were derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP) or 2-aminopyridine (PA), because a few oligosaccharides were likely to be degradated through PA derivatization step. The each derivative was analyzed by reverse-phase high performance liquid chromatography (HPLC) with UV detection. The contents of 2'-FL and LNDFT at Day1 were significantly higher than those of Day 2 and 3. The increase of LNT was observed in Day 3 compared with that in Day 1. These results lead us to conclude that these neutral oligosaccharides were changed by the demand of prebiotics and anti infection of the babies in early lactation.

P202
DPM1, A CATALYTIC SUBUNIT OF DOLICHOL-PHOSPHATE-MANNOSE SYNTHASE, IS TETHERED TO AND STABILIZED ON THE ER MEMBRANE BY DPM3

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Dolichol-phosphate-mannose (Dol-P-Man) is a sole mannosyl donor for the mannosyltransferases within the endoplasmic reticulum (ER), and is required for biosyntheses of *N*-glycan and GPI-anchor precursors, and protein *O*- and *C*-mannoses. Dol-P-Man is synthesized on the cytoplasmic surface of the ER membrane from Dol-P and GDP-Man by Dol-P-Man synthase, and then flipped into the luminal side where the mannosylation occurs. We previously reported that mammalian Dol-P-Man synthase consists of three subunits DPM1, DPM2 and DPM3 (1, 2). In human, deficiency of DPM1, the catalytic subunit of the enzyme, causes congenital disorder of glycosylation type Ie. Mammalian DPM1 has no predicted transmembrane region, therefore it must be tethered to the ER by membrane proteins DPM2 and/or DPM3. To clarify this point, we isolated a DPM3-defective mutant CHO2.38 from various GPI-anchored protein-negative mutant cells. Dol-P-Man synthase activity was not detected in CHO2.38 cells, indicating that DPM3 is essential for the enzyme activity. DPM1 was hardly expressed in CHO2.38 cells, whereas it was expressed well when DPM3 was co-transfected. Lactacystin inhibited the degradation of DPM1 in CHO2.38 cells, suggesting DPM1 was degraded in the cytosol by ubiquitin-proteasome system. Coiled-coil region at C-terminal cytoplasmic tail of DPM3 was important for both tethering and stabilizing DPM1.
*1) Maeda et al., 1998. EMBO J., **17**, 4920-4929; 2) Maeda et al., 2000. EMBO J., **19**, 2475-2482.*

P203
VARIATION IN THE LIPO-OLIGOSACCHARIDE OF *Campylobacter jejuni*: OBSERVATION OF NEW OUTER CORES RESULTING FROM LATERAL EXCHANGES AND SINGLE MUTATIONS.

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The lipooligosaccharide (LOS) of *Campylobacter jejuni* displays considerable variation in the structure of its outer core. Microarray and PCR probing studies have shown that there is extensive variation in the gene content of the locus responsible for the biosynthesis of the LOS. The LOS loci contain from 9 to 19 ORFs and were previously grouped in 7 classes (A to G) based on the organization of 38 distinct genes. While sequencing the LOS biosynthesis locus in *C. jejuni* strains isolated from Guillain-Barré syndrome patients, we observed three additional gene organizations that constitute new classes. The comparison of the gene

organizations in the 10 classes suggests lateral exchange events that resulted in new classes. A single gene exchange event seems to have occurred between the LOS locus of GC149 and a class "A" locus while a similar situation happened between the LOS locus of GB4 and a class "E" locus. A two gene exchange event seems to have occurred between the LOS locus of GB24 and a class "D" locus. We also found strains expressing distinct, but related, outer core structures whose differences can be explained by single mutations. The generation of gene content variability in a specific locus such as the LOS biosynthesis locus provides an example of an adaptive evolution strategy used by a mucosal pathogen to modulate the structure of a cell-surface carbohydrate in order to better survive in a host.

P204

INFLUENCE OF INFLAMMATION ON THE GLYCOSYLATION AND SULFATION OF HUMAN BRONCHIAL MUCINS

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Bronchial mucins from patients suffering from cystic fibrosis (CF) exhibit glycosylation and sulfation alterations in their periphery, especially increased amounts of the sialyl-lewis^x (NeuAcα2-3Galβ1-4[Fucα1-3]GlcNAc-R) and 6-sulfo-sialyl-Lewis^x epitopes that are receptors for Pseudomonas aeruginosa, the bacteria responsible for the early death of CF patients (Scharfman et al., 2001). There is increasing evidence that inflammation affects glycosylation and sulfation of various glycoproteins. Importantly, the amount of sialyl-lewis^x epitopes in bronchial mucins depends on the severity of inflammation, and the pro-inflammatory cytokine TNFα increases the expression and activity of fucosyl-, sialyl- and sulfotransferases involved in the biosynthesis of sialyl-lewis^x and 6-sulfo-sialyl-Lewis^x epitopes (Davril et al., 1999; Delmotte et al., 2002).
In order to better understand the influence of inflammation on the peripheral glycosylation and sulfation of bronchial mucins, we are testing the effect of pro-inflammatory cytokines IL-6 and IL-8 on the expression and activity of glycosyl- and sulfotransferases from human bronchial mucosa explants, by quantitative RT-PCR and Western blotting, respectively.
Quantitative RT-PCR results show that IL-6 increases the expression of the α1,3-fucosyltransferase FUT11, the sialyltransferases ST6Gal-II and ST3Gal-VI, and the sulfotransferases GlcNAc6ST5 and Gal3ST4, whereas IL-8 increases the expression of the α1,3/4-fucosyltransferase FUT3 and the sialyltransferases ST6Gal-II and ST3Gal-VI. Immunoblot analysis show increased amounts of sialyl-lewis^x epitopes in total proteins from bronchial explants treated with IL-6 or IL-8 compared to untreated explants.
We conclude that IL-6 and IL-8 stimulate the expression of fucosyl- (FUT3, FUT11), sialyl- (ST3Gal-VI) and sulfotransferases (GlcNAc6ST5) potentially involved in the biosynthesis of the sialyl-Lewis^x and 6-sulfo-sialyl-Lewis^x épitopes, leading to the increased expression of preferential ligands for P. aeruginosa that could explain the persistence of this bacteria in CF.

This investigation is supported by the Association Vaincre la Mucoviscidose.
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P205

MOLECULAR CLONING AND EXPRESSION OF A NOVEL HUMAN ALPHA2,8-SIALYLTRANSFERASE (HST8SIA VI) RESPONSIBLE FOR THE SYNTHESIS OF THE DISIA MOTIF ON *O*-GLYCOSYLPROTEINS.

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Based on BLAST analysis of the human and mouse genome databases using the human CMP-sialic acid: α2,8-sialyltransferase cDNA (hST8Sia I, EC 2.4.99.8), a putative sialyltransferase gene was identified on human chromosome 10. The genomic organization was found to be similar to that of hST8Sia I and hST8Sia V. Transcriptional expression analysis showed that the newly identified gene was constitutively expressed at low levels in various human tissues and cell lines. We have isolated a full-length cDNA clone from the mammary cell line MCF-7 that encoded a type II membrane protein of 398 amino acid residues with the conserved motifs of sialyltransferases. We established a mammary cell line (MDA-MB-231) stably transfected with the full-length hST8Sia VI and analysis of the sialylated carbohydrate structures expressed at the cell surface clearly indicated the disappearance of Neu5Acα2-3-sialylated structures. The transient expression of a truncated soluble form of the enzyme in either COS-7 cells or insect Sf-9 cells led to the production of an active enzyme whose substrate specificity was determined. Detailed substrate specificity analysis of the hST8Sia VI recombinant enzyme *in vitro,* revealed that this enzyme required the trisaccharide Neu5Acα2-3Galβ1-3GalNAc to generate diSia motif specifically on *O*-glycans.

P206
STRUCTURE AND FUNCTION OF UDP-GLCNAC 2-EPIMERASE/MANNAC KINASE, THE KEY ENZYME OF SIALIC ACID BIOSYNTHESIS, AND ITS ISOFORMS

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Sialylation of glycoproteins and glycolipids on eukaryotic cell surfaces plays an important role during development and regeneration, and in the pathogenesis of diseases. Biosynthesis of sialic acids starts with the action of UDP-GlcNAc 2-epimerase, which converts UDP-GlcNAc to ManNAc and UDP. ManNAc is then phosphorylated by ManNAc kinase, and further metabolized to sialic acids. In mammals both enzymes are joined to the bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNAc kinase (GNE), consisting of an N-terminal epimerase and a C-terminal kinase domain.
Both domains of GNE were expressed separately in insect cells by the baculovirus system. Although it was shown that the single domains were still functional, they revealed drastically reduced enzyme activity and altered oligomeric states (Blume et al. (2004) Biochem. J. 384, 599). Furthermore, reduced stability in absence of the counterpart domain was observed, suggesting a strong intramolecular dependency of the two domains. In conclusion, the two domains of GNE can do work independently, but for optimal enzymatic function the bifunctional structure of the enzyme is required.
Recently, four different splice variants of GNE mRNA were described, revealing different tissue distributions (Watts et al. (2003) Neuromuscul. Disord. 13, 559). These mRNAs encode for three different isoforms of GNE protein with modified N-termini, namely GNE1, GNE2 and GNE3. We expressed these proteins in insect cells and analyzed them for enzyme activities, oligomeric states, regulation and stability. Furthermore, data of recombinant expression of the different GNE isoforms in mammalian cells will be presented and their impact on sialic acid biosynthesis will be discussed.

P207
PHYSIOLOGICAL SIGNIFICANCE OF GPI INOSITOL DEACYLATION IN TRYPANOSOMA BRUCEI

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The structure of glycosylphosphatidylinositol (GPI) anchor is different between higher eukaryotes and protozoans, even in developmental stages of same species. *Trypanosoma brucei* has surface coat consisting of GPI anchored proteins. Bloodstream form, which causes sleeping sickness, is covered by variant surface glycoprotein (VSG) whereas procyclic form, which grows in tsetse fly, is covered by procyclins. During GPI biosynthesis, inositol in PI is acylated. This inositol is deacylated prior to the attachment to VSG in bloodstream form whereas it remains acylated in procyclins. To examine the role of inositol deacylation in trypanosomes, we have identified and characterized a *T. brucei* GPI inositol deacylase (GPIdeAc2) showing predominant expression in the bloodstream form, as expected. Then we examined the roles of GPIdeAc2 in the GPI metabolism. We suppressed GPIdeAc2 expression by RNAi in the bloodstream form. The induction of GPIdeAc2 RNAi led to significant decrease in GPIdeAc2 mRNA level accompanied by accumulation of inositol-acylated glycolipid, indicating that GPIdeAc2 is involved in GPI inositol deacylation. GPIdeAc2 knockdown cells showed a decreased VSG expression level on the cell surface and slow growth rate. Therefore, expression of GPIdeAc2 is very important for bloodstream form. We next induced the overexpression of GPIdeAc2 in procyclic form. GPIdeAc2-overexpressing procyclic form lost the cell surface procyclins due to a release into medium, indicating that suppression of GPIdeAc2 is important for procyclic form. Taken together, our data demonstrated that GPI deacylase activity must be tightly regulated in trypanosome life cycle.

P208
EFFECT OF UV IRRADIATION ON HYALURONAN SYNTHASES IN CULTURED HUMAN EPIDERMAL KERATINOCYTES

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Hyaluronan is a major component of the skin. Although detailed studies on the mechanism of skin disorders caused by UV irradiation and the effects of UV on the hyaluronan molecule have been reported, the gene expression patterns of the enzymes involved in hyaluronan metabolism have not been clarified. Analysis of the genes involved in biosynthesis or degradation of hyaluronan would provide valuable information about the mechanism of photoaging or UV phototherapy. Here we investigated the effect of UV on each

hyaluronan synthase (HAS) expression using cultured normal human epidermal keratinocytes. Cells were cultured for 12 h after irradiation with UVA or UVB. The relative expression levels of HASs were determined by real-time quantitative RT-PCR method. Hyaluronan production in the culture medium was assessed by ELISA-like assay using hyaluronan binding protein. The cultured normal human epidermal keratinocytes express predominantly HAS3 mRNA and also express HAS2. UVB irradiation up-regulated HAS2 and HAS3 while UVA down-regulated HAS3. Hyaluronan production was concomitantly increased by UVB irradiation with the increase in the mRNA level of HASs, suggesting that UV-irradiated cells may enhance production of hyaluronan as one of the defense mechanisms to keep homeostasis.

P209

OCCURRENCE OF β1-3GAL RESIDUES IN *N*-GLYCANS OF INSECT GLYCOPROTEIN, ROYAL JELLY GLYCOPROTEIN: NEW PROCESSING MECHANISM OF N-GLYCOSYLATION IN HONEYBEE CELLS.

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Royal jelly, is one of the most famous health foods, contains several bioactive glycoproteins (RJGP). As a first step to elucidate physiological function of N-glycans on bioactivities of RJGPs, we started glycoform analysis of RJGP.
The *N*-glycans were liberated from the glycopeptides prepared from RJGPs by hydrazinolysis. After *N*-acetylation, the sugar chains were coupled with 2-aminopyridine. The PA-sugar chains were purified by RP-HPLC and SF-HPLC. The structures of the PA-sugar chains were determined by combination of glycosidase digestions, ESI-MS, methylation analysis, and 1H-NMR.
In this paper, we report the occurrence of β 1-3 galactosyl residues in *N*-glycan moiety of RJGP, suggesting that honeybee cells have ability to biosynthesize β 1-3gal-containing complex type *N*-glycans. Since it has been considered that insect cells can produce only high-mannose type and β 1-3/6 fucose-containing paucimannose type *N*-glycans, our finding was the first example to indicate that honeybee cells posses a set of glycosyltransferase genes (GN-T, β 1-3Gal-T) required for construction of complex type *N*-glycans and can be used for production of bioactive glycoproteins bearing the β1-3Gal-containing *N*-glycans. We also revealed that the β 1-3Gal-containing *N*-glycans are linked to N (28) in a sequence of G(24)-E-S-L-N-K(29) of 350 kDa RJGP (apisin). Based on the primary structures, we have proposed a new processing pathway for insect *N*-glycans. 1) Kimura, Y., et al. *Biosci. Biotechnol. Biochem.,* **67**, 1852-1856 (2003). 2) Kimura, M., et al., *Biosci. Biotechnol. Biochem.,* **67**, 2055-2058 (2003).

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Occurrence of β 1-3Gal residue in N-glycans of insect glycoprotein, royal jelly glycoproteins: New processing mechanism of N-glycosylation in honeybee cells.

P210

DETERMINATION OF SULFATIDE-SPECIES FROM RAT RENAL TUBULE CELLS BY MALDI-TOF MS.

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Sulfatides from small amount of rat renal tubule cells were prepared and analyzed together with their lyso-forms by MALDI-TOF MS. Sulfatide SM4s (sulfated galactosylceramide), SM3 (sulfated lactosylceramide) and SM2 (sulfated gangliotriaosylceramide) were clearly detected with negative mode analysis. Major m/z of SM4s were 878.6, 892.6 and 906.6, whereas those of SM3 were 1042.7, 1056.7 and 1070.7 and those of SM2 were 1245.8, 1259.8 and 1273.8 respectively. Major m/z of LysoSM4s was 540.3, whereas those of LysoSM3 and LysoSM2 were 720.3 and 923.4 respectively. These results indicated that the SM4s were composed of species of d18:1 (sphingosine)-C22:0h (C22 hydroxy fatty acid), d18:1-C23:0h, and d18:1-C24:0h, whereas the SM3/SM2 were composed of species of t18:0 (phytosphingosine) -C22:0 (non-hydroxy fatty acid), t18:0-C23:0, and t18:0-C24:0, respectively. In addition, significant amount of uncommon sphingoid of C-20 dihydrosphingosine were observed in SM3 and SM2. The difference of the composed ceramides between SM4s and SM3/SM2 may be reflected by the topologic difference of glycosyltransferases. Namely SM4s is derived from galactosylceramide synthesized by ceramide galactosyltransferase located in the endoplasmic reticulum, whereas SM3/SM2 are derived from glucosylceramide synthesized by ceramide glucosyltransferase whose catalytic site is located at the outer surface of the Golgi. Nevertheless, the different series of sulfatides SM4s and

SM3/SM2 utilized common length of both fatty acids and sphingoids, although they are oppositely hydroxylated. Since glycosphingolipids are critical element of the microdomain, the lengths and hydroxylations of fatty acids and sphingoids in the sulfatides might be sophisticatedly regulated to effectively constitute the microdomain.

P211
ROLE OF MDR-1 IN GLYCOLIPID BIOSYNTHESIS PROVIDES NEW APPROACH FOR CANCER TREATMENT, AND FABRY AND GAUCHER DISEASES

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MDR-1 multidrug resistance protein is a major cause of cancer treatment failure. This energy-dependent drug efflux pump is reversed by cyclosporin A (CsA). MDR-1 is a Golgi glucosylceramide (GlcCer) translocase providing glycosphingolipid (GSL) precursor inside the Golgi for neutral GSL synthesis (*J Biol Chem* 275:6246 and 279:2867). CsA prevents neutral GSL synthesis in all cells tested except HeLa. After transfection with *uog*1, a C18 ceramide synthase, subsequent HeLa C18 GSL synthesis is CsA sensitive, indicating both MDR1 dependent and independent mechanisms for GlcCer translocation.
Globotriaosylceramide (Gb_3) and MDR1 are partially cell surface colocalized and the effect of GSLs analogs on MDR was determined. Treatment with adamantylGb_3 (adaGb_3) reversed the cellular MDR phenotype, similar to CsA, suggesting that adaGb_3 could be an inhibitor of MDR1. AdaGb_3 inhibition of MDR1-mediated drug efflux supports this possibility.
CsA inhibits neutral GSL synthesis and thus is a candidate treatment for certain GSL storage diseases. The effect of CsA on the recovery of Gb_3 plasma levels was studied in a Fabry's mouse model after treatment with α-galactosidase therapy. Immunohistochemistry of frozen sections, and lipid extraction and thin layer chromatography of tissues of CsA-treated and untreated mice were performed. Initial promising results indicate that CsA reduce the accumulation of Gb_3 in plasma and in liver. CsA treatment of Gaucher's lymphoblasts also showed markedly reduced GlcCer compared with untreated lymphoblasts. These studies highlight the importance of MDR1 in GSL biosynthesis and new avenues for the manipulation of MDR1 in the management of clinical diseases.

P212
CHEMICAL AND GENETIC STUDIES OF THE O-ANTIGENS OF *SHIGELLA BOYDII* AND THEIR RELATIONSHIP TO THE O-ANTIGENS OF *ESCHERICHIA COLI*

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Bacteria *Shigella*, a cause of shigellosis and diarrhea, show a high-level housekeeping genes sequence similarity to *Escherichia coli* (Ec). These bacteria are classified by O-antigens, which represent the lipopolysaccharide O-chains. There are 33 distinct O-antigen gene clusters in *Shigella*, including 18 in *Shigella boydii* (Sb). From them, O-antigen structures are known in 12 Sb clones, that of Sb type 8 being identical to the Ec O143 O-antigen structure. Now we report on the remaining Sb O-antigens and compare O-antigens of Sb and Ec.
Using NMR spectroscopy and chemical methods, unique O-antigen structures were established for Sb types 16-18, whereas Sb types 1 and 3 were found to share O-antigens with Ec O149 and O167, respectively. O-antigen gene clusters sequencing and serological data were in consistence with the chemical data. They also suggested that six more Sb types (2, 4, 5, 11, 14 and 15) have O-antigens in common with Ec O87, O53, O79, O105, O32 and O112, respectively. Therefore, nine Sb O-antigens are shared by nine Ec clones and the other nine are unique to *Shigella* clones.
The identical O-antigen gene clusters may have been assembled in one strain from each Sb and Ec pair and then spread to the other. It is thought that *Shigella* evolved about 10,000 years ago after the development of agricultural settlements. The expansion of the O-antigen diversity over such a short period indicates its importance for *Shigella*.This work was supported by RFBR grants 03-04-39020 and 05-04-48992, grant NSh-1557.2003.3 and NSFC General Program 30270029.

P213
GLYCOLIPID CHANGES IN GalCer SULFOTRANSFERASE-DEFICIENT MICE: A SINGLE ENZYME CATALYZES THE SYNTHESIS OF VARIOUS SULFOGLYCOLIPIDS

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Sulfoglycolipids are specifically expressed in the myelin sheath, spermatozoa, renal tubular cells, and the gastrointestinal tract. The mouse kidney contains GalCer I^3-sulfate (SM4s) as well as LacCer II^3-sulfate (SM3) and Gg_4Cer II^3,IV^3-bis-sulfate (SB1a), whereas the intestine expresses Gg_4Cer IV^3-sulfate (SM1b) and SB1a. Insight into the function of these sulfoglycolipids has been provided by gene-targeted disruption of two enzymes which catalyze synthesis of SM4s. Ceramide galactosyltransferase (CGT) catalyzes synthesis of GalCer, and GalCer sulfotransferase (CST) sulfates GalCer to SM4s. Both CGT- and CST-deficient mice manifest neurological disorders caused by myelin dysfunction and male infertility due to the arrest of spermatogenesis. However, apparent functional defects were not observed in the kidney and intestine. Since Cgt and Cst transcripts are normally detected in the kidney and intestine, glycolipids of these organs were analyzed in CST-deficient mice. As expected, homozygous mutant mice did not express SM4s. In addition, SM3, SM1b and SB1a were completely absent in the kidney and intestine. These data provide the first definitive evidence that the Cst gene is also responsible for biosynthesis of sulfoglycolipids with longer carbohydrate chains *in vivo*. In place of SM4s, its precursor GalCer increased in the kidney, while Gg_4Cer, the precursor of SM1b, increased in the intestine of CST-deficient mice. Among major gangliosides, an increase of GM1 was prominent. Given that GM1, SM1b and SB1a share the same ganglio-series biosynthetic pathway, the increase of GM1 should be a compensatory process, which may partly contribute to the normal function of kidney and intestine in CST-deficient mice.

P214
THE LIPID REMODELING OF GPI-ANCHORED PROTEINS IN MAMMALIAN CELLS

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We established a novel type of mutant CHO cell lines, in which the biosynthesis of glycosylphosphatidylinositol-anchored proteins (GPI-APs) is normal but GPI-APs are secreted to culture medium following the cleavage of GPI between inositol and phosphate. We identified the responsible gene for these mutant cells, PGAP2 (Post GPI-Attachment to Proteins 2), which is a previously uncharacterised Golgi-localized protein. By structural analysis of intracellular GPI-APs, we found that two events caused the secretion of GPI-APs in PGAP2 mutant cells. GPI-APs were cleaved by a phospholipase A_2 (PLA_2) like enzyme in the Golgi/TGN resulting in formation of lyso-GPI-APs which, in turn, became substrates for an unknown lyso-phospholipase D enzyme on the cell surface. GPI-APs are synthesized from phosphatidylinositol (PI) in the ER. Most PIs of GPI-APs on the cell surface are composed of saturated fatty acids, whereas major free PIs have a highly unsaturated fatty acid at the sn-2 position, suggesting that the lipid remodeling of GPI might occur during the transport. Therefore, we hypothesized that the PLA_2-like enzyme catalyzes such remodeling. To test our hypothesis, we established double mutant CHO cells from PGAP2 mutant cells where the surface expression of GPI-APs was recovered, and investigated the lipid composition of the PI of GPI-APs. By MS/MS analysis we confirmed that the PI of GPI in the double mutant cells had a highly unsaturated fatty acid unlike wild-type cells. These results indicate that GPI in mammalian cells is remodeled in the Golgi by a PGAP2-containing enzyme.

P215
GDP-L-FUCOSE METABOLISM IN FIBROBLASTS FROM LAD II/CDG IIC PATIENTS

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LAD II/CGD IIc is a rare genetic disease due to a reduction of fucose in glycoconjugates. The molecular basis has been identified in a defective uptake of GDP-L-fucose into the Golgi and at least four different mutations of the transporter have been identified. GDP-L-fucose biosynthesis occurs in the cytosolic compartment through a “de novo” pathway, starting from GDP-D-mannose, and a “salvage” one, which recycles free fucose.
To elucidate the effect of a reduced uptake into the Golgi on GDP-L-fucose metabolism, we analyzed GDP-L-fucose cytosolic concentration and the activity of its biosynthetic

pathways in fibroblasts from two LAD II patients bearing different mutations of the Golgi transporter. Interestingly, GDP-L-fucose cytosolic levels resulted highly reduced in LAD II compared to controls. This result was unexpected since a decreased influx into the Golgi would suggest increased or, at least, normal cytosolic levels of GDP-L-fucose. "In vitro" enzymatic activity, expression and sequence of all the enzymes involved in GDP-L-fucose biosynthesis resulted comparable to control fibroblasts, thus excluding a down-regulation or an additional mutation of these proteins. Conversely, an increased degradation of GDP-L-fucose was observed in cell lysates from LAD II cells. This activity was found to be associated to membranes and it was not specific for GDP-L-fucose, but it was able to degrade also other nucleotide-sugars. No activity was found in the cytosolic fraction. These data suggest the presence of a regulatory mechanism which controls GDP-L-fucose cytosolic levels. Experiments are in progress to identify this mechanisms and to determine its role in LAD II.

P216
REGULATORY MECHANISM OF INFLAMMATION-INDUCED EXPRESSION OF β4-GALACTOSYLTRANSFERASES IN HUMAN ENDOTHELIAL CELLS

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TNFα is one of the mediators that induce glycan-dependent leukocyte-endothelial interactions at sites of inflammation. Recently, we have reported [1] that TNFα also increases the expression of 19 glycosylation-related genes in human endothelial cells (HUVEC), affecting especially those involved in the expression of immunologically important epitopes, like 6-sulfo-sialyl-Lewis^X (the L-Selectin ligand), 6'-sialyllactosamine (the CD22 ligand), and polylactosamine (one of the galectin ligands). β4GalT-1 was one of the glycosyltransferases that was affected by TNFα. We now show that TNFα-induced stabilization of β4GalT1 mRNA mediated solely through the second AU-rich element (AU2) in the 3'-untranslated. Under basal conditions the destabilizing factor TTP was bound tot AU2 resulting in rapid mRNA turnover. Stimulation by TNFα resulted in the dissociation of TTP from its binding site paralleled by an increase in the β4GalT1 mRNA half-life. Two signaling pathways appeared to be involved; one mediated by IKKβ and the other by PKCδ [2].
This mechanism of regulation could be shared by two other members of the β4GalT family, β4GalT-5 and -6, which also were upregulated in HUVEC under the influence of TNFα. Interestingly, other glycosylation-related genes that were modulated by TNFα in HUVEC also carried AU-rich elements in their 3'-UTR, indicating that this could be a general mechanism of regulation.
1. García Vallejo et al. J Cell Physiol (2005) in press.
2. Gringhuis et al. Mol Cell Biol (2005) in press.

P217
GLYCOSYLATION IN BRAIN METASTASIS.

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We evaluated the glycosylation pattern of 7 different cancer metastasis. Paraffin embedded tissues were analyzed by co-localization with lectins and antibodies by fluorescence microscopy. The lectins were ALL (*Amaranthus leucocarpus*: Galβ-1,3GalNAcα-1,0-Ser/Thr), PNA (*Arachis hypogaea*; Gal-α1,4-GlcNAc), SNA (*Sambucus nigra;* Neu5Acα-2,6-Gal/Gal), MAA (*Maackia amurensis*; Neu5Acα-2,3-Gal/Gal), MRL (*Macrobrachium rosenbergii*; Neu5,9Ac2), LPA (*Limulus polyphemus;* Neu5Ac) and ConA (*Cannavalia ensiformis*; α-D-Man). Neuronal or glial cells identified with Synaptophysin, GFAP (Glial fibrillary acidic protein), NCAM (Neural cell adhesion Molecule). The lung cancer metastasis was mainly identified by PNA, followed by ALL and SNA. The kidney metastasis were mainly recognized by MRL, PNA, and SNA. The papillary metastasis expressed MRL and SNA carbohydrates-linked. The non-Hodgkin tissues were MRL, ConA and PNA positive more than MAA and SNA. The breast metastasis were mainly recognized by ALL and ConA positive. Thyroid metastasis was recognized by ALL, MRL, PNA, SNA and ConA in a regular form. Malign melanomas expressed a less amount of LPA and PNA reactive carbohydrates. We found a differential pattern of glycoconjugates in metastasis; moreover, distant implantation of tumoral cells seems to be an addressed event mediated by oligosaccharidic expression.
Financed in part by CONACyT and FUNSALUD, México.

P218
FREE AMINO ACIDS ARE HALLMARK FOR FRESHWATER PRAWN DEFENSE MECHANISMS AND METABOLISM.

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The hemolymph of juveniles and adult crustaceans participated in adaptation and as regulators of the defense mechanisms have been suggested. In *M. rosenbergi* juvenile organisms, the main free sugar was GlcNAc and its concentration was 2.4-fold higher than glucose ($P<0.05$) and significantly higher than other sugar residues ($P<0.01$), moreover; in contrast to adults, most of the sialic acid identified is free in the hemolymph. Hemolymph's free components from both adults and juveniles inhibit the hemagglutinating activity of the lectin, specific for N-acetylated sugar residues. Adult organisms showed proline and alanine as the main FAA; but in juveniles, glycine represented the most important FAA ($P<0.01$); moreover, juveniles inhibited the production of superoxide radicals by hemocytes ($p<0.05$) as compared to adult. Suggesting that the concentration of glucogenic AA, and carbohydrates in *M. rosenbergii* seems to be influenced by the maturation process; furthermore, due to the high proportion of free GlcNAc and glycine, they seems to participate as precursors for metabolism and regulating their humoral and cellular defense mechanisms, respectively.

This project has been financed in part by PAPIIT-UNAM and the program Cuerpos Académicos del PIFI 3.0 UAEMorelos, México.

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FREE N-ACETYL-GLUCOSAMINE AND GLUCOGENIC AMINO ACIDS ARE HALLMARK FOR *MACROBRACHIUM ROSENBERGII* (CRUSTACEA DECAPODA) DEFENSE MECHANISMS AND METABOLISM.

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The hemolymph of juveniles and adult crustaceans participated in adaptation and as regulators of the defense mechanisms have been suggested. In *M. rosenbergi* juvenile organisms, the main free sugar was GlcNAc and its concentration was 2.4-fold higher than glucose ($P<0.05$) and significantly higher than other sugar residues ($P<0.01$), moreover; in contrast to adults, most of the sialic acid identified is free in the hemolymph. Hemolymph's free components from both adults and juveniles inhibit the hemagglutinating activity of the lectin, specific for N-acetylated sugar residues. Adult organisms showed proline and alanine as the main FAA; but in juveniles, glycine represented the most important FAA ($P<0.01$); moreover, juveniles inhibited the production of superoxide radicals by hemocytes ($p<0.05$) as compared to adult. Suggesting that the concentration of glucogenic AA, and carbohydrates in *M. rosenbergii* seems to be influenced by the maturation process; furthermore, due to the high proportion of free GlcNAc and glycine, they seems to participate as precursors for metabolism and regulating their humoral and cellular defense mechanisms, respectively.

This project has been financed in part by PAPIIT-UNAM and the program Cuerpos Académicos del PIFI 3.0 UAEMorelos, México.

Glycoconjugates in cell signalling and cell surface interactions

P220
OVEREXPRESSION OF α-FUCOSYLTRANSFERASE-VII MODIFIES THE SIGNAL TRANSDUCTION OF INSULIN RECEPTOR

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In order to study the function of fucosyl residue on surface glycans, the cDNA of α1,3FucT-VII was transfected into human H7721 hepatocarcinoma cell line, which expressed low level of α1,3FucT-VII. The expression of sialyl Lewis X (SLe^x), the product of α1,3FucT-VII, both on the surface and the α-subunit of insulin receptor (InR) but not on EGF receptor, was increased after the transfection of α1,3FucT-VII. The tyrosine auto-phophorylation of InR β-subunit and insulin substrate-1 (IRS-1) as well as the Ser/Thr phophorylation at T308 and S473 of protein kinase B (PKB), novel protein kinase (PKN) and p42/44 MAPK (mitogen activated protein kinase) and MEK (MAPK kinase) were up-regulated. On the other hand, the expressions of some signaling proteins were increased (phosphatidylinositide dependent kinase-1, PKN, Raf), unchanged (InR, PKB, p42/44 MAPK and MEK) or decreased (IRS-1). Overexpression of α1,3FucT-VII also significantly promoted the expression of β-catenin protein and the activity of its down stream transcription factor TCF. The magnitude of the above alterations was in proportional with the expression of α1,3FucT-VII mRNA in the cells and SLe^x on the surface or InR. In addition, the antibody of SLe^x and the inhibitor of InR tyrosine phosphorylation could obviously attenuate or abolish the difference in the phosphorylation of various signaling molecules in transfected cells with different expression of α1,3FucT-VII. These results indicated that addition of fucose residues on cell surface glycan may alter the signal transduction from InR. However, other mechanisms not involving the surface receptor could not be ruled out.

P221
LOW-POTASSIUM INDUCED APOPTOSIS IN RAT CEREBELLAR NEURONS IS ACCOMPANIED BY THE REORGANIZATION OF LIPID MEMBRANE DOMAINS

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Introduction. Lipid membrane domains in neurons provide a microenvironment within the plasma membrane for reciprocal interaction of functional significance between lipid and protein molecules involved in several aspects of neuronal signal transduction, including survival and apoptosis pathways.
Experimental approaches. Granule neurons prepared from the cerebella of 8-days old rats were differentiated in vitro in the presence of 25 mM K^+. Apoptosis was induced by lowering the K^+ concentration to 5 mM for different times. The lipid and protein compositions of detergent-resistant ganglioside-enriched membrane fractions from control and apoptotic neurons were analyzed.
Results. During apoptosis induced by low potassium concentration in rat cerebellar granule cells, ceramide is produced by sphingomyelin hydrolysis due to sphingomyelinases activation. This event leads to a deep change in the cell lipid composition, that is specifically restricted to the lipid membrane domains. The protein pattern associated with the sphingolipid-enriched membrane fraction undergoes dramatic changes during low potassium-induced apoptosis. In particular, the amount of cellular prion protein (PrP) and of c-Src and Fyn tyrosine kinases associated with this fraction is much lower in apoptotic neurons than in control cells. Remarkably, the neural cell adhesion molecule TAG-1 and the Src-family tyrosine kinase Lyn did not change in the sphingolipid-enriched membrane fraction during neuronal apoptosis. Conclusions. These data suggest that PrP and TAG-1, specifically associated with different Src family protein tyrosine kinases, belong to different signaling cassettes within sphingolipid-enriched domains from rat cerebellar neurons, the former being possibly involved in the control of neuronal survival.

P222
TARGETED DISRUPTION OF N-ACETYLGLUCOSAMINYLTRANSFERASE GNT-VB IN HUMAN NEUROBLASTOMA CELLS ELEVATES THE EXPRESSION OF β1 INTEGRIN LEADING TO IMPAIRED NEURITE OUTGROWTH, INCREASED ADHESION, AND REDUCED RATES OF MIGRATION ON LAMININ.

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Neuroblastoma is a childhood malignancy associated with a high rate of metastasis and a poor clinical prognosis. We are investigating the role of the N-acetylglucosaminyl- transferase known as GnT-VB (GnT-IX) in neuroblastoma metastasis. Using RNA interference, we have established neuroblastoma cell lines with reduced GnT-VB expression. Neuroblastoma cells with reduced GnT-VB expression display increased adhesion, reduced migration, and impaired neuritogenesis when plated on the extracellular matrix protein laminin. Expression of a recombinant active form of GnT-VB that is not targeted for RNA interference silencing can reverse these changes indicating the direct involvement of GnT-VB in mediating these phenotype changes. To better understand the mechanisms regulating these changes in adhesion and migration, we examined the expression levels of various laminin receptors, and find that GnT-VB deficient cells express altered levels of integrins compared to control cells. GnT-VB is capable of performing both N-linked and O-linked glycosylation. To confirm that the changes in cell adhesion and neurite extension for GnT-VB silenced cells were due to O-linked glycosylation, we silenced the enzyme, POMGnT1, that is required before GnT-VB can add β(1,6) GlcNAc to O-linked substrates. Reduced expression of POMGnT1 leads to a phenotype similar to that seen for cells with reduced GnT-VB and confirms that these phenotype changes are due to O-linked mannose glycosylation. Taken together, our results suggest that O-linked glycosylation of glycoprotein acceptors by GnT-VB may contribute to the decreased adhesion and increased rates of metastasis in human neuroblastoma. Therapies targeted at the inhibition of GnT-VB expression may be useful for the prevention of neuroblastoma metastasis.

P223

CD43 CAPPING AS A CARBOHYDRATE-MEDIATED CELL-CLEARANCE SIGNAL OF APOPTOTIC AND NON-APOPTOTIC CELLS

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Eearly apoptotic Jurkat cells undergo transient capping of CD43 glycoprotein, and its sialylpolylactosaminyl saccharide chains serve as ligands for macrophage recognition and phagocytosis (1). This apoptotic stage is earlier than phosphatidylserine (PS) exposure and the exposed PS-mediated macrophage recognition (2). Here, we show that induction of CD43 capping on Jurkat cell surface without causing apoptosis also leads to the carbohydrate-mediated macrophage recognition.
Treatment of Jurkat cells with adequate concentrations of cytochalasin B resulted in CD43 capping on the cell surface. The CD43-capped cells were susceptible to macrophage recognition. This recognition was inhibited by anti-CD43 antibody, and by sialylpolylactosamine-containing oligosaccharides. The cytochalasin B-treated cells were not apoptotic as judged by TUNEL staining, PS-exposure, and caspase activities. A broad caspase inhibitor Z-VAD-fmk did not block the cytochalasin B-induced CD43 capping and the macrophage recognition. A calcium ionophore A23187 also induced CD43 capping and the subsequent macrophage recognition without causing apoptosis. These results indicate that CD43 capping is sufficient to lead to the carbohydrate-mediated macrophage recognition, and cell death is not prerequisite for the recognition. It is thus conceivable that even alive cells are recognized and removed when they undergo CD43 capping by nonapoptotic signals.

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P224

ALTERED METABOLISM AND MEMBRANE ORGANIZATION OF GLYCOSPHINGOLIPIDS IN FENRETINIDE-RESISTANT HUMAN OVARIAN CARCINOMA CELLS.

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introduction. in the case of antitumor drug resistance, the role of complex glycosphingolipids has never been systematically investigated, even if it might be crucial in defining the properties of drug resistant tumor cells, thus a prime target for strategies aimed to overcome this phenomenon. in the present work, we studied the sphingolipid patterns, metabolic pathways and membrane organization in human ovarian carcinoma cells characterized by different sensitivity to antitumor drugs. *experimental approaches.* we used two drug-resistant cell lines derived from the parental line a2780. 2780ad doxorubicin-resistant cells are a classical mdr tumor cell line. a2780/hpr are hpr-resistant cells, not cross-resistant to other drugs. the sphingolipid patterns were analyzed by metabolic labeling with radioactive sphingosine. the activities of key enzymes in sphingolipid metabolism were assayed in cell free lysates using radioactive substrates. the mrna levels of glccer synthase, gm3 synthase, gm2 synthase and caveolin-1 were assessed by quantitative real time pcr. *results*. we

found similar neutral glycosphingolipid levels and higher sphingomyelin levels in resistant 2780ad cells respect to those in parental cells. in a2780/hpr cells, gm3 synthase was markedly overexpressed, leading to gm3 and gm2 levels 5-fold higher than in sensitive parental cells and laccer levels proportionally lower. we have also found that a2780/hpr cells overexpress caveolin-1, a membrane adapter usually enriched within specialized sphingolipid-enriched membrane domains. *conclusions.* these results indicated that different forms of drug resistance are characterized by specific alterations in the patterns of complex cell glycosphingolipids, possibly linked to a different supermolecular membrane organization.

P225
IN VIVO GROWTH ADVANTAGE OF COLON CANCER CELL LINES EXPRESSING β-GALACTOSIDE α2,6 SIALYLTRANSFERASE (ST6Gal.I)

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The addition of sialic acid in α2,6-linkage to lactosaminic termini of glycoproteins is mainly mediated by β-galactoside α2,6 sialyltransferase (ST6Gal.I). This enzyme and its cognate oligosaccharide structure are frequently overexpressed in cancer and are associated with increased malignancy but the cellular and molecular bases of this relationship are not clear. In this study, we have investigated the role of ST6Gal.I in the *in vivo* growth. First, we have xenografted in nude mice colon cancer cell lines and analyzed the expression of ST6Gal.I and of α2,6-sialylated sugar chains in the xenograft-derived (XD) cell lines. Compared with parental cell lines, the XD cell lines express dramatically increased levels of ST6Gal.I mRNA, detected by RT-PCR analysis, and enzyme activity, as well as an increased reactivity with the α2,6-sialyl-specific lectin from *Sambucus nigra* (SNA) suggesting either a selection of the cells expressing higher levels of ST6Gal.I. Then, we investigated the *in vivo* growth of clones derived from transfection with the ST6Gal.I cDNA of the colon cancer cell line SW48, which was originally devoid of ST6Gal.I expression. Compared with mock-transfectants, ST6Gal.I-transfectants form more rapidly growing tumours. Moreover, when an identical number of mock- and ST6Gal.I-transfected cells was mixed and injected in the nude mice, the xenografts and the xenograft-derived cell lines were comprised only of SNA-positive ST6Gal.I-transfectants, while mock-transfected cells were usually not present in the xenograft. These results indicate that during the growth of the tumour the cells expressing high levels of α2,6-sialylated glycoconjugates are positively selected because of a growth advantage.

P226
MECHANISMS FOR ACCELERATED CELL GROWTH AND INVASION ACTIVITY WITH GD3 EXPRESSION IN HUMAN MELANOMAS

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Introduction : Disialoganglioside GD3 is specifically expressed on human melanoma cells, and has been considered to serve as an important factor in the malignant phenotypes. In this study, we analyzed the mechanisms by which GD3 induces malignant properties of human melanoma cells using GD3 synthase cDNA-transfectant cells.
Methods : We established GD3-expressing melanoma cell lines by introducing GD3 synthase cDNA into a GD3-deficient melanoma cell line, SK-MEL-28-N1. The proliferation was evaluated by MTT assay, and invasion potential was by Boyden-chamber invasion assay. The expression levels of MMP family members were analyzed by RT-PCR. The MMP activity was measured by gelatin zymography.
Results and discussion : GD3-positive transfectant cells showed increased proliferation and invasion. They showed enhanced tyrosine-phosphorylation of p130Cas and paxillin after stimulation with FCS. The knock-down of these molecules with siRNAs indicated that p130Cas is involved in proliferation and invasion, and paxillin is involved in invasion in GD3-expressing cells. Treatment of GD3-expressing cells with MAPK inhibitor U0126 did not clearly affect the tyrosine phosphorylation of p130Cas and paxilline. In contrast, wortmannin treatment of them resulted in partial reduction of the tyrosine phosphorylation of the two molecules, suggesting important roles of PI3-kinase in the malignant phenotypes. Whether MMP2 and/or MMP9 can be induced with FCS stimulation in GD3-expressing cells is now under investigation.

P226b
CORE FUCOSYLATION REGULATES TGF-BETA1 RECEPTOR-MEDIATED SIGNALING

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αlpha1,6-Fucosyltransferase (Fut8) catalyzes the transfer of a fucosyl moiety from GDP-fucose to the innermost GlcNAc residue of hybrid and complex N-linked oligosaccharides on glycoproteins via αlpha1,6-linkage to form core fucosylation.

Disruption of Fut8 gene exhibits emphysema-like changes in mouse lung. In order to investigate the molecular mechanism involved, we established mouse embryonic fibroblast (MEF) of Fut8+/+ and Fut8-/-, which derived from wild-type and Fut8-null mice, respectively. As expected, the core fucosylation was completely blocked in Fut8-/-MEFs. TGF-βeta1 receptor-mediated signals are important for controlling cell growth and differentiation, and has also marked effects on ECM homeostasis. The deletion of Fut8 does not alter IL-1βeta receptor-mediated function, but diminishes TGF-βeta1-mediated MMPs production. The binding ability of 125I-TGF-βeta1 was significantly reduced in Fut8-/- cells compared with Fut8+/+ cells, which could be rescued by reintroducing Fut8 to Fut8-/- cells. Consistent with this, Smad2 phosphorylation, a down-stream factor of TGF-βeta1 was dramatically suppressed in Fut8-/- cells, which was recovered by reintroducing Fut8 to Fut8-/- cells. Taken together, these results demonstrate that core fucosylation has an important role in the regulation of TGF-βeta1 receptor function. We therefore, assume that Fut8-/- lungs are committed to overexpressing MMPs, probably because they escape from the TGF-βeta1 suppressor mechanism, which operates in wild-type lungs, although other functions of core fucosylation of N-glycan-bearing glycoproteins might also be involved in the development of emphysema of lung.

P227
LIPID RAFT IS INVOLVED IN THE ENHANCED PHOSPHORYLATION OF P130CAS AND PAXILLIN INDUCED BY GANGLIOSIDE GD3 IN MALIGNANT MELANOMA CELLS

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Purpose: Ganglioside GD3 is expressed in neuro-ectoderm-derived malignant tumors such as human melanomas. Recently, we reported that GD3 promotes cell growth and invasion through p130Cas and paxillin in malignant melanoma cells. However the mechanism by which lipid raft contributes in the phosphorylation of p130Cas and paxillin induced by GD3 in malignant melanoma cells has not been clearly shown. In the present study, we analyzed a potential role of lipid raft in the enhanced tyrosine phosphorylation of p130Cas and paxillin with GD3 expression. Methods: After the transfection of SK-MEL-28-N1 cells with GD3 synthase cDNA, $GD3^+$ transfectant cells were established. Raft fractionation was done using Triton X-100 lysates and sucrose density gradient ultracentrifugation, and immunoblotting was performed with anti-adaptor protein antibodies and an anti-phosphotyrosine antibody. MβCD was used to examine a potential role of lipid raft in the enhanced tyrosine phosphorylation of p130Cas and paxillin. Results and Discussion: Raft fractionation of Triton X-100 extracts after FCS treatment revealed that majority of tyrosine-phosphorylated bands of p130Cas and paxillin were present in non-raft fractions. However, a strong band of tyrosine-phosphorylated paxillin was found at raft fractions. When lipid raft was disrupted using 1% MβCD, the tyrosine phosphorylation levels of p130Cas and paxillin at the time point of 25 min were clearly and significantly reduced in cells treated with MβCD than in untreated cells, suggesting important roles of lipid raft in the signal enhancement with GD3. Intracellular localization of GD3, and GD3-associating molecules are now under investigation.

P228
SIALIC ACID PRECURSORS ARE TOOLS TO INTERFERE WITH WITH POLYSIALYLATION OF THE NEURAL CELL ADHESION MOLECULE IN VITRO AND IN VIVO

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Sialic acid (Sia) is expressed as terminal sugar in many glycoconjugates and plays an important role during development and regeneration. Addition of homopolymers of Sia (polysialic acid = polySia) is a unique and highly regulated posttranslational modification of the neural cell adhesion molecule (NCAM). The presence of polySia affects NCAM-dependent cell adhesion and plays an important role during brain development, neural regeneration and plastic processes including learning and memory. Polysialylated NCAM is expressed on several neuroendocrine tumors of high malignancy and correlates with poor prognosis. Two closely related enzymes, the polysialyltransferase ST8SiaII and ST8SiaIV, catalyze the biosynthesis of polySia. This review summarizes recent approaches to modify the degree of polySia on NCAM in vitro and in vivo. First, we describe the selective inhibition of ST8SiaII using synthetic sialic acid precursors. Second, we demonstrate that the key enzyme of the sialic acid biosynthesis (UDP-N-acetylglucosamine 2-epimerase = GNE) regulates and limits the synthesis of polysialic acid.

P229
NOVEL OLIGOSACCHARIDES HAVE INHIBITORY ACTIVITIES ON PROLIFERATIONS OF HUMAN LEUKEMIA CELLS

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It has been reported that glucosamine ($GlcNH_2$) is distributed in connective and cartilage tissues of human and other animals. It is well known that this amino sugar is a precursor substance of glycosaminoglycans, and it has suppression activity of nitric oxide synthesis and reparation effect of arthrodial cartilage such as osteoarthritis. Thus applications of glucosamine are noticed for drugs and foods.
We report here that novel oligosaccharides containing $GlcNH_2$ inhibit the proliferation of human leukemia K562 and BALL cell but not human normal umbilical cord fibroblast (HUC-F2) suggesting that human leukemia cell has unique receptor for novel oligosaccharides.
Lactosamine, allolactosamine, N-acetylmelibiosamine, melibiosamine (MelNH2), galactosyl-N-acetyllactosamine and galactosyllactosamine were synthesized by reverse reactions of α- and β-galactosidases of bacteria, and FITC-labeled melibiosamine (MelNH-FITC) was synthesized.
Each cell suspension (0.36 ml) of K562, BALL and HUC-F2 (about 4.5 ~ 8.8 X 10^4 cells/ml) was added to a 24 well plate, and incubated with 1-10 mM saccharides for 3 - 4 days. Cell counts were done using a hemocytometer. Cells were stained by Cellstain Hoechist 33258 (H33258) for apoptosis and by MelNH-FITC.
It was observed that novel oligosaccharide had inhibitory activities on leukemia cell proliferations, and that the apoptosis of K562 cell was induced by addition of $MelNH_2$. When MelNH-FITC was added to the control K562 cell culture, the cells were not stained, but wounded or dead cells by addition of $MelNH_2$ were stained. It is suggested that the K562 cell has a unique and important receptor for $MelNH_2$ and MelNH-FITC.

P230
THE C-MANNOSYLATED TETRAPEPTIDE MODULATES LIPOPOLYSACCHARIDE SIGNALING IN MACROPHAGE-LIKE RAW264.7 CELLS

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C-mannosylation is a novel type of glycosylation in proteins. There are several examples of proteins in which the specific motif W-X-X-W is mannosylated at the first W to produce C-mannosylated tryptophan (CMW). Although C-mannosylation modifies W-X-X-W, predicted to be a functional motif of various integral proteins such as thrombospondin, the biological relevance of C-mannosylation in the cell is still not known. In this study, to investigate biological function of C-mannosylation in cellular signaling, we examined the effect of CMW and C-mannosylated tetrapeptides on lipopolysaccharide (LPS)-signaling pathway in macrophage-like RAW264.7 cells. RAW264.7 cells were stimulated with LPS in the presence or absence of synthesized chemicals (e.g., W-S-P-W, C-mannosylated W-S-P-W, and CMW), then the activation status of LPS signaling was estimated by examining the phosphorylation status of several downstream kinases by immunoblot analysis using specific antibodies. Although LPS activated several mitogen-activated protein kinases such as ERK, p38 MAPK, and Jun N-terminal kinase, their activation was apparently enhanced in the presence of C-mannosylated W-S-P-W. However, LPS-induced phosphorylation of IL-1 receptor-associated kinase 1 (IRAK1), the most upstream kinase of LPS signaling, showed no difference in the cells treated with or without C-mannosylated W-S-P-W. Furthermore, the binding of LPS-FITC to the cells was not influenced even in the presence of C-mannosylated W-S-P-W. These results indicate that C-mannosylated W-S-P-W enhances LPS signaling in RAW264.7 cells not by its direct influence on LPS-TLR4 complex on cell surface but by unidentified mechanism of the molecule, suggesting a novel biological function of C-mannosylated motif in glycoproteins.

P231
N-GLYCANS OF BETA-PROPELLER DOMAIN OF ALPHA5 INTEGRIN ARE ESSENTIAL FOR ALPHA5BETA1 HETERODIMER FOMATION AND ITS BIOLOGICAL FUNCTIONS

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N-glycosylation on integrin alpha5beta1 is thought to play crucial roles in cell spreading, cell migration, its ligand binding as well as dimerization, but the underlying mechanism remains unclear. To investigate importance of N-glycans of this integrin in detail, we have carried out sequential site-directed mutagenesis to remove 14 putative N-glycosylation sites on alpha5 integirn. These mutants were transfected and expressed in CHO-B2 cells, an integrin alpha5-deficient chinese hamster ovary cell line. Removal of putative N-glycosylation sites on beta-propeller, Thigh, Calf-1 and Calf-2 domains of alpha5 subunit resulted in decreased molecular weight estimated by SDS-PAGE, suggesting that all of those domains carry N-glycans. Importantly, mutation of triple N-glycosylation sites (3, 4 and 5) on blade 4 of beta-propeller of alpha5 subunit, but not other putative N-glycosylation sites, significantly suppressed cell adhesion to fibronectin, focal adhesion kinase phosphorylation and hetero-dimerization. Conversely, integrin alpha5 subunit carrying only these three potential N-glycosylation sites had comparable activity for cell spreading. Taken together, these results strongly suggest that hetero-dimerization and functions of alpha5beta1 integrin are essentially required for N-glycosylation on blade 4 of beta-propeller of alpha5 subunit.

P232

GANGLIOSIDE GM3 MODULATES TUMOR SUPPRESSOR PTEN-MEDIATED CELL CYCLE PROGRESSION; TRANSCRIPTIONAL INDUCTION OF $P21^{WAF}$ AND $P27^{KIP1}$ BY INHIBITION OF PI-3K/AKT PATHWAY

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The simple ganglioside GM3 has been shown to have antiproliferative effects in several *in vitro* and *in vivo* cancer models. Although the exogenous ganglioside GM3 has an inhibitory effect on cancer cell proliferation, the exact mechanism by which it promotes cell cycle arrest remains unclear. Previous studies showed that MDM2 is an oncoprotein that controls tumorigenesis through both p53-dependent and -independent mechanisms, and tumor suppressor PTEN, a dual specificity phosphatase that antagonizes phosphatidylinositol 3-kinase (PI-3K)/AKT signaling, is capable of blocking MDM2 nuclear translocation and destabilizing the MDM2 protein. Results from our current study show that GM3 treatment dramatically increases not only the PTEN expression but also cyclin-dependent kinase (CDK) inhibitor (CKI) $p21^{WAF1}$ expression through the accumulation of p53 protein by the PTEN-mediated inhibition of the PI-3K/AKT/MDM2 survival signaling in HCT116 colon cancer cells, confirming that ganglioside GM3 functions as the p53 protein stabilizer. Moreover, the data herein clearly show that ganglioside GM3 induces p53-dependent transcriptional activity of $p21^{WAF1}$, as evidenced by the $p21^{WAF1}$ promoter-driven luciferase reporter plasmid (full-length $p21^{WAF1}$ promoter and a construct lacking the p53 binding sites). Additionally, ganglioside GM3 enhances expression of CKI $p27^{kip1}$ through the PTEN-mediated inhibition of the PI-3K/AKT signaling. Furthermore, the down-regulation of the cyclin E and CDK2 was clearly observed in GM3-treated HCT116 cells, but the down-regulation of cyclin D1 and CDK4 was not. These results suggest that ganglioside GM3-stimulated PTEN expression modulates cell cycle regulatory proteins, thus leading to cell cycle arrest. We conclude that ganglioside GM3 represents a modulator of cancer cell proliferation and may have potential for use in colorectal cancer therapy.

P233

CO-EXISTENCE OF THE α2,9- AND α2,8-LINKED POLYSIALIC ACIDS ON SEA URCHIN SPERM AND THEIR DIFFERENTIAL ROLES IN FERTILIZATION

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The α2,8- and α2,9-linked polyNeu5Ac structures are known to occur in capsular polysaccharides of certain pathogenic bacteria. In vertebrates, several glycoproteins like neural cell adhesion molecule contain the α2,8-linked polyNeu5Ac, and only two glycoproteins are shown to contain α2,9-linked polyNeu5Ac [Miyata *et al.* (2004) *Glycobiology* 14, 827; Inoue *et al.* (2002) *J. Biol. Chem.* 278, 85411]. Recently, we demonstrated the presence of 8-*O*-sulfated Neu5Ac-capped α2,9-linked polyNeu5Ac in flagellasialin, a major sialoglycoprotein of sea urchin sperm flagellum. In this study, we cloned cDNAs for flagellasialin from three sea urchin species. Flagellasialin is a novel glycoprotein consisting of 96 amino acids and a single transmembrane segment, and highly conserved among sea urchin species. Flagellasialin is a Thr-rich, heavily *O*-glycosylated glycoprotein with a diverse molecular mass of 40-80 kDa. In addition to the α2,9-linked polyNeu5Ac, we also demonstrated the presence of the α2,8-linked polyNeu5Ac in sea urchin sperm using highly sensitive chemical and immunochemical methods. The α2,8-linked polyNeu5Ac was detected in glycolipids as well as in a 190 kDa-glycoprotein, a membrane glycoprotein different from flagellasialin. The α2,8-linked polyNeu5Ac is present in both sperm head and flagellum, while the α2,9-linked polyNeu5Ac was exclusively localized in sperm flagellum. Furthermore, we showed that the antibody specific to the α2,9-linked

polyNeu5Ac inhibited fertilization and sperm motility, while the antibody recognizing α2,8-linked polyNeu5Ac had no effect on fertilization. These results thus indicated that these two polyNeu5Ac structures are expressed on distinct glycoproteins on the same sperm and have differential roles in fertilization.

P234
IN SITU VISUALIZATION OF THE BINDING OF THE CYTOPLASMIC/NUCLEAR TOBACCO LECTIN TO ENDOGENOUS N-GLYCANS

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Tobacco (*Nicotiana tabacum*) leaves react upon treatment with the plant hormone jasmonate by the accumulation of a cytoplasmic/nuclear lectin (Chen et al., 2002). Preliminary specificity studies using surface plasmon resonance experiments and hapten inhibition assays indicated that *N. tabacum* agglutinin (called Nictaba) specifically interacts with oligomers of N-acetylglucosamine. However, analysis of lectin binding on glycan arrays containing 190 different glycans revealed that the lectin does not have the highest affinity for chito-oligosaccharides but exhibits a strong binding to high mannose as well as multiple biantennary N-glycans carrying terminal Gal, GlcNAc, and GalNAc. A detailed analysis of the binding studies suggests that the binding site of Nictaba is most complementary to Manβ1-4GlcNAcβ1-4GlcNAcβ-N-Asn.
To check whether Nictaba interacts *in vivo* with endogenous glycans a GFP-Nictaba fusion protein was expressed in tobacco BY-2 cells and its location studied by confocal microscopy. *In vitro* binding studies confirmed that the fusion protein expressed in BY-2 cells possesses carbohydrate-binding activity. Microscopic analyses confirmed the previously reported cytoplasmic/nuclear location and, in addition, revealed that the fusion protein 'stains' the cytoplasm-exposed surface of many subcellular structures, indicating that they are covered with glycoproteins protruding into the cytoplasm. Western blot experiments with crude extracts and purified nuclei from BY-2 cells confirmed that Nictaba interacts in a chito-oligosaccharide inhibitable way with numerous proteins including most nuclear proteins. Hence, the observed *in situ* binding of the GFP-Nictaba fusion protein is at least partly due to a specific interaction with endogenous glycoprotein receptor molecules.

Chen, Y., Peumans, W.J., Hause, B., Bras, J., Kumar, M., Proost, P., Barre, A., Rougé, P., Van Damme, E.J.M., 2002. Jasmonic acid methyl ester induces the synthesis of a cytoplasmic/nuclear chitooligosaccharide-binding lectin in tobacco leaves. FASEB J. 16, 905-907.

P235
THE MEMBRANE ENVIRONMENT OF ENDOGENOUS CELLULAR PRION PROTEIN IN PRIMARY RAT CEREBELLAR NEURONS.

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Introduction. Regions highly enriched in sphingolipids and characterized by a limited but selected content in protein molecules have been detected in cell membranes. They are particularly enriched in GPI-anchored proteins and acyl-linked non-receptor tyrosine kinases, attached to the external and the inner leaflet of the plasma membrane respectively. The role of this organization in supermolecular units has been only partially understood. In this work we studied the membrane environment of cellular prion protein in primary cultured rat cerebellar neurons differentiated *in vitro*.
Experimental approaches. A detergent-resistant, low-density membrane fraction (DRM) was separated by flotation on sucrose gradient from rat cerebellar granule cells differentiated in vitro. The lipid and protein compositions of this ganglioside-enriched membrane fraction were analyzed. Prion protein was separated from DRM by a monoclonal antibody immunoseparation procedure under experimental conditions designed to preserve lipid-mediated membrane organization.
Results. In differentiated neurons, about 45% of total cellular prion protein (corresponding to a 35-fold enrichment) is associated with DRM, from which it was efficiently separated by immunoseparation procedure. Several proteins were found in the prion protein-enriched membrane domains (i.e. the non receptor tyrosine kinases Lyn and Fyn and the neuronal GPI-anchored protein Thy-1). These domains contained as well about 50% of the sphingolipids, cholesterol and phosphatidylcholine present in DRM. All main sphingolipids, including sphingomyelin, neutral glycosphingolipids and gangliosides, were similarly enriched in the prion protein-rich membrane domains.
Conclusions. Prion protein environment in neuronal plasma membrane resulted to be a complex entity, whose integrity requires a network of lipid-mediated non-covalent interactions.

P236
THE EXPRESSION OF Sd^a β1,4 N-ACETYLGALACTOSAMINYLTRANSFERASE INHIBITS SIALYL LEWIS X EXPRESSION IN COLON CANCER CELLS

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The biosynthesis of the Sd^a histo-blood group antigen [Siaα2,3(GalNAcβ1,4)Galβ1,4GlcNAc-R], is catalyzed by Sd^a β1,4GalNAc transferase (β1,4GalNAc-T). This enzyme, which catalyzes the addition of GalNAc, the immunodominant sugar, to the Gal residue of an α2,3-sialylated type 2 chain, is highly expressed in normal colon but it is dramatically downregulated in colon cancer. The acceptor structure of β1,4GalNAc-T is also a precursor of the biosynthesis of the sialyl Lewis x (sLex) antigen [Siaα2,3Galβ1,4(Fuca1,3)GlcNAc-R], a major ligand for adhesion molecules of the selectin family whose ectopic expression in cancers favours metastasis formation. The fact that the precursor structures of the Sd^a and the sLex antigens are identical suggests a competition in the biosynthesis of the two structures and that the downregulation of β1,4GalNAc-T opens the way to the biosynthesis of sLex, favouring the metastatic progression of colon cancer. The two β1,4GalNAc-T mRNA species cloned to date differ in the first exon and predict two polypeptides with a different cytoplasmic tail: one encodes a polypeptide with an exceptionally long cytoplasmic sequence of 67 aminoacids, the other encodes a polypeptide with a cytoplasmic tail of 7 residues. To investigate the relationship between β1,4GalNAc-T and sLex expression, we have stably expressed the two forms in the human colorectal cancer cell line LS174T, which expresses good level of sLex. Compared with mock-transfectants, the clones expressing either form of β1,4GalNAc-T show a dramatic downregulation of sLex expression in FACS analysis, demonstrating that the level of expression of β1,4GalNAc-T modulates the biosynthesis of sLex, *in vitro*.

P237
CHITOSAN-BASED SCAFFOLD MATERIAL COVALENTLY CONJUGATEING BFGF HAS AN IMPROVED CELL PROLIFERATION ACTIVITIES

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Introduction: Signaling molecules such as growth factors are essential for tissue repair or regeneration. Although some materials were prepared by mixing growth factors to scaffold materials, they had difficulties in controlling the release of growth factors and in maintaining the activities. We hypothesized that it resolved the problems to prepare the scaffold using covalent bond. Therefore, we developed the chitosan-based material covalently conjugating bFGF, which had a significant cell proliferation activity.
Experimental approaches: Chitosan solution and 2-iminothiolane were added to potassium phosphate buffer, and kept for 3 h at room temperature in a nitrogen atmosphere. Heparin and bFGF were dissolved in Tris-HCl, and kept for 2 h. Chitosan derivative and bFGF-heparin derivative were mixed with dimethyl sulfoxide for 4 h in a nitrogen atmosphere. A disulfide bond was formed between the SH group of chitosan derivative and cysteine residues of bFGF-heparin derivative.
Cell proliferation assay was carried out for evaluating the material. It was placed to a 96-well plate and incubated. Chondrocytes and fibroblasts were seeded and incubated for 24 h at 37 °C. WST-8 was added and incubated for 2 h. The absorbance was measured at 490 nm with microplate reader. The obtained data were analyzed using ANOVA and Fisher's PLSD test.
Results: The material showed significantly improved cell proliferation activities of chondrocytes and fibroblasts.
Conclusions: We prepared a chitosan-based scaffold material covalently conjugating bFGF. Because it had the excellent cell proliferation activities of chondrocytes and fibroblasts, it was thought to have a great potential for enhancing tissue regeneration.

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PROTEIN C-MANNOSYLATION IN MONOCYTE DIFFERENTIATION

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In C-mannosylation of glycoproteins, the specific motif W-X-X-W is mannosylated at the first tryptophan. Although C-mannosylation modifies W-X-X-W, a predicted functional motif of various integral proteins, the physiological relevance of C-mannosylation in cells is still unknown. In this study, to investigate the relationship between C-mannosylation and monocyte differentiation, we examined the alteration of C-mannosylated proteins in the cells under differentiation. Human promonocytic cell lines (THP-1 and U937) were treated with phorbol-12-myristate-13-acetate (PMA) to get differentiated into macrophage-like cells. C-mannosylated proteins were examined by immunoblot analysis and immunofluorescence microscopy using antibodies against C-mannosylated tryptophan, and the activity of C-mannosyltransferase was also assayed in cell lysates from differentiated and undifferentiated cells. In differentiated cells, the level of cellular C-mannosylation was increased in the proteins corresponding to 240, 207, 185 and 104 kDa. C-mannosylated proteins were detected mainly in the secretory granules in the cells, and also detected in culture medium from the differentiated cells. In spite of the increase of C-mannosylated proteins, no significant increase was observed in the C-mannosyltransferase activity in cell lysates from differentiated cells compared with undifferentiated ones. Collectively, these results indicate that elevation of the level of protein C-mannosylation is highly associated with the differentiation of monocytes by PMA, suggesting C-mannosylation as a novel marker for the monocyte differentiation. Although the increase of protein C-mannosylation was not simply explained by the change of C-mannosyltransferase activity, it might partly be due to the PMA-induced increase of some target proteins to be C-mannosylated.

P239
FUNCTIONAL ASPECT OF SCAVENBER RECEPTOR ON TUMOR GROWTH

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Scavenger receptor (group A, type I, II) is expressed on the cell surface of monocytes/macrophages and recognizes a molecule carring a specific pattern of anionic charges. Previously we found that mucins produced by epithelial cancer cells bound to the scavenger receptor, leading to induction of cyclooxygenase 2 (COX2) and subsequent elevation of PGE2 production (Proc Natl Acad Sci USA 100: 2736-2741, 2003). It is generally agreed that PGE2 plays various biological roles in immune-regulation, angiogenesis and inhibition of apoptosis. Mouse mammary tumor cell line has two sublines; TA3-Ha (mucin-producing cell) and TA3-St (mucin-non producing cell). Subcutaneous tumor of TA3-Ha grew faster than TA3-St tumor did. In the former tumor tissues, mucin, COX2 infiltrated macrophages and angiogenesis were found more prominently than these in the latter tissues. Next, we prepared recombinant soluble scavenger receptor (sSCR) and sSCR cDNA-transfected TA3-Ha cell. sSCR could bind to mucin and poly I but not to poly C. The sSCR cDNA and mock transfectants were subcutaneously injected into mice. The tumor of mock transfectant grew much faster than that of the sSCR cDNA transfectant. Furthermore, TA3-Ha cells (1×10^3 cells) or mixture of sSCR cDNA or mock transfectant (1×10^3 cells) with TA3-Ha cell (1×10^3 cells) were subcutaneously injected into mice. The tumor grew in the order of mock transfectants plus TA3-Ha cells, TA3-Ha cells, and sSCR cDNA transfectants plus TA3-Ha cells. These results suggest that ligation of mucins to SCR on infiltrated macrophages may be relevant to tumor growth.

P240
OVEREXPRESSION OF CAVEOLIN-1 ATTENUATES SIGNALS BY ALTERING RAFT-LOCALIZATION OF GANGLIOSIDE GD3 IN MALIGNANT MELANOMA CELLS

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Purpose: Caveolin-1 (Cav-1) is the primary structural component of caveolae and is implicated in the processes of vesicular transport, cholesterol balance, transformation, and tumorigenesis. Recently we reported that melanoma-associated ganglioside, GD3 promotes cell growth through p130Cas and paxillin in malignant melanoma cells. However, relationship between Cav-1 and GD3 has not well understood. In the present study, we analyzed effects of Cav-1 overexpression on the cell phenotypes and biochemical features of lipid raft. Method: SK-MEL-28 cells were transfected with a Cav-1 expression vector, resulting in the establishment of Cav-1 expressing (Cav-1$^+$) cell lines. Raft fractionation was done using triton X-100 lysates and sucrose density gradient ultracentrifugation. Results and Discussion: Compared with vector control (Cav-1$^-$) cell lines, Cav-1$^+$ cells exhibited reduced cell proliferation and cell motility. In

accordance with the reduced cell growth, Cav-1^{+} cells showed less activation of MAP kinase and Akt than Cav-1^{-} cells. Furthermore, phosphorylation levels of p130Cas and Paxillin decreased in Cav-1^{+} cells. Then intracellular localization of GD3 was analyzed, showing that majority of GD3 bands were found in raft fractions in Cav-1^{-} cells . In contrast, GD3 was widely distributed inside and outside of raft fraction in Cav-1^{+} cells. These results suggest that overexpression of Cav-1 alters the intracellular localization of GD3 inside/outside raft fraction, and down-regurates signal intensities for the malignant phenotypes of melanoma cells. Now, Cav-1-associating molecules are under investigation.

P241
BINDING OF RHAMNOSE-BINDING LECTIN TO Gb3 CAUSES EARLY APOPTOTIC STATUS TO BURKITT'S LYMPHOMA CELLS

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Silurus asotus (catfish) egg lectin (SAL) has potent affinity to Gal α-linked carbohydrate chains of not only glycoproteins but also glycosphingolipids such as globotriaosylceramide (Gb3). SAL uniformly bound to surfaces of Gb3-expressing Burkitt's lymphoma cells, such as Raji and Daudi cells, and strongly agglutinated these cells. On the other hand, Gb3 molecules were sprinkled over the surfaces of Gb3-expressing cells. After a short period treatment of Raji and Daudi cells with SAL, each cell size of the cells was 10 and 25% smaller than that of untreated cells, respectively. Treatment of Gb3-expressing cells with SAL caused increase in binding of annexin V and incorporation of propidium iodide, however, neither caspase-8 and -3 activation nor DNA fragmentation was observed after treatment of SAL for 22 h. Since SAL did not induce cell death to Gb3-expressing cells, SAL may function as an inducer of early apoptotic signal. We have revealed that SAL did not bind to D-PDMP-treated Raji cells, and no cell shrinkage was observed in the Gb3-deficient Raji cells by SAL, indicating that Gb3 localized in glycosphingolipid-enriched microdomain (GEM) was involved in SAL-induced cell shrinkage, and that the glycoprotein ligands on Gb3-deficient Raji cells for SAL were not included in this phenomenon. These results suggest that SAL leads the cells to early apoptotic status via binding to Gb3 existing in GEM, and that this binding is prerequisite condition to induce early stage of apoptosis.

P242
INTERACTIONS OF GALECTIN-3 WITH LAMININ-5 AND THE METASTASIS ASSOCIATED PROTEIN C4.4A

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C4.4A is a N- and O-glycosylated GPI-anchored protein with low homology to urokinase receptor (uPAR). In normal tissues expression of C4.4A is restricted to the basal layer of epidermis, the epithelial layer of oesophagus and to uterine gland and placenta. It is intriguing that C4.4 A is highly upregulated in different cancers like melanoma, breast, lung and pancreatic cancer. There is evidence that C4.4A expression may be induced or upregulated during tumor progression and wound healing. In line with the involvement of C4.4A in these processes is our recent finding that C4.4A binds laminin-1 and -5 thereby promoting cell adhesion and migration. Recently, we have also identified galectin-3, a carbohydrate-binding protein, as a C4.4A ligand. Galectin-3 binds to different cellular receptors, like integrins, and extracellular matrix components like laminin-1 and fibronectin. Galectin-3 is involved in cell adhesion and migration and like C4.4A is upregulated in different cancers and in wound healing.
The C4.4A-glycosylation profile differs between cancer cell lines. Using a GST pull down assay with recombinant galectin-3 we could show here that the C4.4A-glycosylation profile of cancer cells directly affects the C4.4A-galectin-3 interaction.
In addition, using surface plasmon resonance technique, we found that the two C4.4A ligands galectin-3 and laminin-5 are able to directly interact with each other. Binding of galectin-3 to laminin-5 was found to be similar to galectin-3-fibronectin binding.
Interactions between all three proteins C4.4A, galectin-3 and laminin-5 and their modulation via different C4.4A-glycosilations may play an important role in re-epithelisation and cancer progression.

P243
CHARACTERIZATION OF THE uPAR PLASMA MEMBRANE DOMAIN

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The urokinase plasminogen activator receptor (uPAR) is a glycosylphosphatidyl-inositol (GPI) anchored protein. In the cells, uPAR partitions in both detergent-resistant (DRMs,

highly enriched of glycosphingolipids) and detergent-soluble (DS) membranes. The presence of uPAR in DRMs vs. DS raises the question of whether its functions are affected by the glycolipid environment. Indeed, it has been described that uPAR interaction with VN and the uPAR cleavage by uPA occur preferentially in DRMs where uPAR dimers are mostly present.
The aim of the study was to identify and characterize the plasma membrane glycosphingolipid domain containing uPAR. We isolated from HEK 293-uPAR cells the DRM fraction by sucrose density gradients in 1% Triton X-100 and analysed its lipid and protein composition. While the DRMs contained 40% of the membrane sphingolipids (SL) and 25% of cholesterol, the protein content of these fractions amounted to only 1% of total cell protein and to less than 10% of phosphatidylethanolamine, PE.
About 30% of membrane uPAR and 15% of caveolin-1, but no TfR, partitioned to this cholesterol and SL-enriched membrane fraction. When uPAR was immunoprecipitated from these fractions, under conditions that maintain the integrity of membrane domain, only 15% of the SL and 3% of total proteins present in the fraction co-immunoprecipitated with uPAR. Qualitatively, the glycolipid composition of the uPAR-specific immunoprecipitates resulted strongly impoverished of the neutral glycosphingolipids and enriched in gangliosides. Caveolin was not component of the uPAR environment.

P244
IMPORTANCE OF GALACTOFURANOSE FOR THE EUKARYOTIC HUMAN PATHOGEN ASPERGILLUS FUMIGATUS

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D-Galactofuranose is an unusual sugar present at the cell surface of many pathogenic bacteria and essential for their viability or pathogenicity. It is for instance indispensable for the cell viability of *Mycobacteria*. Importantly, galactofuranose is not present in human; therefore the molecules involved in its synthesis represent attractive targets for the development of new antimicrobial drugs. So far, studies concentrated on bacteria but galactofuranose is often found in important surface molecules of protozoan parasites and fungi such as the human pathogen *Aspergillus fumigatus*. The latter creates invasive aspergillosis in immunocompromised patients, which is a leading cause of infectious death in modern hospitals. In contrast to *Mycobacteria*, the importance of galactofuranose for fungi is completely unknown because none of the enzymes involved in galactofuranose metabolism had been characterized. We succeeded in identifying and characterizing the first eukaryotic UDP-galactopyranose mutase (UGM), an enzyme that plays a central role in galactofuranose biosynthesis by providing UDP-galactofuranose to various galactofuranosyltransferases. The enzyme has been cloned from the opportunistic fungus *Aspergillus fumigatus* and its activity demonstrated by an *in vivo* complementation system and an *in vitro* assay. A mutant deficient in galactofuranose was then created by targeted replacement of UGM gene in *Aspergillus fumigatus* D141. The absence of galactofuranose in the deletion mutant was confirmed by various techniques. Although the mutant is viable, its radial growth is decreased. Moreover it exhibits a reduced virulence compared to wild type in a murine infection model.

P245
DISCOVERY OF POLYSIALIC ACID RESIDUES ON MICROGLIA CELLS

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Polysialic acid (polySia), a polymer of sialic acid, is known to occur in capsular polysaccharides of neuroinvasive bacteria such as *Neisseria meningitidis* group B and *Escherichia coli* K1, salmonid fish egg polysialoglycoprotein, vertebrate brain neural cell adhesion molecule (NCAM), and voltage-gated sodium channels of electroplax and rat brain. The polySia on NCAM is considered to be a regulatory molecule for cell-cell/extracellular matrix interactions in brain via its anti-adhesive effect. Recently, we identified CD36 as a third polySia-containing glycoprotein member in mammals. CD36 is a member of the SR-B class of the scavenger receptor superfamily and occurs not only in milk, but also in platelet and some type of macrophages. Interestingly, CD36 was polysialylated in milk and mammary gland, although not in platelet. To extend our knowledge on the presence of polySia residues on CD36, we focused on microglia cells that express CD36 in brain. We used the primary microglial culture cells from mouse brain and a microglial cell line. We first discovered the presence of polySia residues on microglia cells using anti-polySia antibody and endo-*N*-acylneuraminidase. We also demonstrated that the polySia residues are linked to *N*- and *O*-linked glycan chains in NCAM and CD36, respectively, by immunoprecipitation experiments. Furthermore, we showed polySia residues on microglia cells rapidly disappeared on the LPS-stimulated activation. This is

the first demonstration of the occurrence of polySia on microglia cells, and of rapid disappearance of polySia in biological processes.

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ROLE OF GANGLIOSIDES IN THE ASSOCIATION OF ERBB2 WITH LIPID RAFTS IN MAMMARY EPITHELIAL HC11 CELLS

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Gangliosides are not uniformly distributed within the outer leaflet of the plasma membrane, but they segregate, together with cholesterol, GPI-anchored proteins and signaling transduction molecules like tyrosine-kinase receptors (RTKs), into unique more or less stable clusters or microdomains indicated as “glycosphingolipid-enriched domains” (GEM), that contribute to the membrane structure, organization, and function. Indeed, GEM are viewed as dynamic and preferential association of sphingolipids and cholesterol into moving platforms, which can selectively incorporate or exclude proteins and contribute to lipid–mediated protein trafficking and signal transduction.

In this study we analyzed the role of gangliosides in the association of the ErbB2 RTK with lipid rafts in mammary epithelial HC11 cells. Scanning confocal microscopy experiments revealed a strict ErbB2-GM3 co-localization in wild-type cells. In addition, the analysis of membrane fractions obtained by linear sucrose gradient showed that ErbB2, as well as EGFR and Shc-p66 (proteins correlated with the ErbB2 signal transduction pathway), were preferentially enriched in lipid rafts together with gangliosides.

The block of endogenous ganglioside synthesis by [D]-PDMP induced a drastic cell surface redistribution of ErbB2, EGFR and Shc-p66, within the Triton-soluble fractions, as revealed by linear sucrose gradient analysis. This redistribution was partially reverted when exogenous GM3 was added to ganglioside depleted HC11 cells.

These results point out the key role of ganglioside GM3 in retaining ErbB2 into lipid microdomains.

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CELL DENSITY-DEPENDENT CHANGES IN THE EXPRESSION OF GALECTIN-3 IN MOUSE BALB/3T3 CELLS

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Our previous study showed that the β-1,4-galactosylation of N-linked oligosaccharides of mouse Balb/3T3 cells increases significantly at higher cell density as revealed by lectin blot analysis using RCA-I. This suggests that the β-1,4-galactosylated N-linked oligosaccharides are important for cellular interactions occurred at higher cell density. To examine this further, we looked for a receptor for the galactose using an asialo-transferrin-Sepharose column, and isolated galectin-3 as a major galactose-binding protein from high density cells as revealed by MALDI-TOF mass spectrometric analysis. Western blot analysis of the whole cell lysates showed that the total amount of galectin-3 increases in Balb/3T3 cells at 100% in density, as compared with the cells at 20% in density. The immunocytochemical study showed that galectin-3 is present at cell surface of higher density cells, while not of lower density cells in which it stays within cells. When the cells at higher density were treated with jack bean β-galactosidase, more amounts of 5-bromo-deoxyuridine were incorporated into the cells, suggesting that cell growth is induced by the galactosidase digestion. These results indicate that both galactose residues and galectin-3 are involved in the cellular interaction occurred at higher cell density. Currently, the vascular cell adhesion molecule (V-CAM)-1 was isolated as one of the galectin-3 binding proteins from higher density cells. Whether the binding of galectin-3 to V-CAM-1 is involved in the growth control remains to be elucidated.

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INTERACTION OF TETANUS NEUROTOXIN WITH GANGLIOSIDES

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Tetanus toxin is a very potent neurotoxin produced by *Clostridium tetani* . Potency is derived from high affinity for nerve terminal receptors leading to retrograde transport to spinal cord neurons where it blocks neurotransmitter release. The carboxyl terminal third of the toxin folds into a receptor

binding domain (Hc). Neuronal cell receptors are thought to comprise several molecules in which polysialylated gangliosides play an important role. Our research efforts are to define the sites on the toxin important for binding to polysialylated gangliosides and to determine which carbohydrate structures interact with the toxin. We show by mutagenesis that residues W1289, D1222, H1271, and R1226 are important for binding to the ganglioside GT1b. Since R1226 is located away from the other residues this result supports the proposal that polysialylated gangliosides interact at two sites on the Hc domain of tetanus toxin.
To determine which oligosaccharide structures interact with the toxin, the Hc- domain was screened against a solid phase array of 181 biotinylated oligosaccharides. Of these structures only 5 were identified with significant binding. All of these oligosaccharides structures were related to gangliosides with exception of the disaccharide, Galβ1-4GlcNAcβ–. The binding was quantitated by surface plasmon resonance. The Hc domain was also screened against a printed array containing 200 oligosaccharides covalently attached to a solid phase. The printed array confirmed the binding to GD2, GD3 and GT3. Our results suggest that carbohydrate receptors of tetanus toxin may be limited to ganglioside related structures.

P249
GANGLIOSIDE-MEDIATED INHIBITION OF AXON REGENERATION: STRUCTURE-FUNCTION RELATIONSHIP

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Myelin associated glycoprotein, MAG, is a member of the Siglec family of sialic-acid binding lectins. MAG is expressed in the central and peripheral nervous systems, where it is postulated to stabilize axon-myelin interactions. MAG also inhibits axon regeneration at sites of central nervous system injury. The functions of MAG are postulated to be dependent on its binding to the prominent axonal gangliosides, GD1a and GT1b, each of which carries a "NeuAc α2-3 Gal β1-3 GalNAc" terminus. Blocking MAG binding to gangliosides, biochemically or genetically, attenuates its biological functions *in vitro* and *in vivo*. MAG, on myelin, may initiate transmembrane signaling by inducing the clustering of axonal gangliosides. In support of this hypothesis, multivalent clustering of GD1a or GT1b using specific monoclonal antibodies inhibits axon outgrowth from rat cerebellar granule neurons (CGN), whereas clustering of GM1 is without effect. Pretreatment of CGN with sialidase converts cell surface GD1a and GT1b to GM1, and abolishes axon outgrowth inhibition by anti-GD1a or anti-GT1b. However, following sialidase treatment, addition of pre-clustered anti-GM1 antibody inhibits axon outgrowth. Mice with a disrupted *Galgt1* gene (GM2-synthase) do not express GD1a and GT1b or other complex gangliosides, but instead overexpress GD3 and GM3. Axon outgrowth from *Galgt1*-null mice CGN is not inhibited by antibody-induced clustering of GD1a or GT1b (which are absent), nor are they inhibited by clustering GD3 (which is overexpressed). These results imply that (i) different gangliosides associate with different effector molecules, and (ii) specific ganglioside-association allows for specific function. Supp. by NIH.

P250
Gb3/CD77-MEDIATED APOPTOSIS: FURTHER CHARACTE-RIZATION OF THE SIGNALING PATHWAYS INDUCED BY ANTI-Gb3 mAb AND VEROTOXIN-1

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Globotriasosylceramide (Gb3), a neutral glycosphingolipid, is the B-cell differentiation antigen CD77 and acts as the receptor for most Shiga toxins including verotoxin 1 (VT-1).

We have shown previously that two ligands of this antigen (anti-Gb3/CD77 mAb and VT-1) induce apoptosis of Burkitt's lymphoma cells by completely distinct pathways. VT-1 triggers a caspase- and mitochondria-dependent pathway whereas anti-Gb3/CD77 mAb trigger an apoptotic pathway which is not mediated by caspases activation but involve ceramide accumulation and is inhibited by antioxidant compounds. We have now further deciphered these two mechanisms. We show that during VT-1-induced apoptosis: 1) Bid and Bax, two bcl-2 family members, are translocated to mitochondria; 2) c-$FLIP_L$, an inhibitor of caspase-8, is rapidly degraded via the proteasome system; 3) proteasome inhibitors protect cells from apoptosis. One can therefore assume that in Burkitt's cells c-$FLIP_{L,}$ and caspase 8 are present as heterodimers and that VT-1 induces degradation of c-$FLIP_L$ which allows activation of caspase 8. When activated, caspase 8 cleaves cytoplasmic Bid which is then relocated to the mitochondria and participates, with Bax, in the mitochondrial membrane permeabilization. Concerning anti-Gb3/CD77 mAb-induced apoptosis, we report that: 1) among various antioxidants compounds, only N-acetyl-cysteine and reduced glutathione (GSH) are able to inhibit apoptosis; 2) no decrease in intracellular GSH concentration is detected; 3) an early but low generation of reactive oxygen species (ROS) occurs; 4) a moderate inhibition of the antioxidant enzyme activities is observed only in certain cell lines. Further studies are therefore needed to determine the exact characteristics of the oxidative stress involved in anti-Gb3/CD77 mAb-induced apoptosis.

P251
EPIDERMAL GROWTH FACTOR-INDUCED MOBILIZATION OF A GANGLIOSIDE- SPECIFIC SIALIDASE (NEU3) TO MEMBRANE RUFFLES

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Human ganglioside-specific sialidase, NEU3, is localized at cell membranes and is thought to be involved in regulation of various biological processes at cell surfaces through modulation of gangliosides (1-3).
We here explored functional subcellular localization of the sialidase by immunofluorescence and found accumulation at leading edges of cell membranes in the presence of serum in culture. In response to epidermal growth factor (EGF), the sialidase redistributed rapidly to ruffling cell membranes of squamous carcinoma-derived A431 cells, and co-localized with Rac-1, a small G protein participating in actin reorganization and cell motility. Overexpression of NEU3 enhanced tyrosine-phosphorylation of the epidermal growth factor receptor (EGFR) as well as promoting Rac-1 activation and cell migration. NEU3 was co-immunoprecipitated by anti-EGFR antibody, and EGF-stimulation yielded a higher amount of NEU3 in the immunoprecipitates. These results indicate that NEU3 is able to mobilize to membrane ruffles in response to growth stimuli and activate the Rac-1 signaling, probably through promotion of EGFR phosphorylation, leading to increased cell motility.

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P252
INTERLEUKIN-6 CARBOHYDRATE RECOGNITION TRIGGERS PHYSIOLOGICAL ACTIVITY

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Various cytokines have carbohydrate recognition abilities, which seem to modulate the interaction between cytokines and their receptors in immune system. We previously reported that the dual recognition of IL-2 to the specific peptide sequence and high-mannose type glycan on the IL-2 receptorα συβυνιτ (IL-2Rα) triggers the high-affinity complex formation of IL-2/IL-2Rαβγ. Using the plate coated with various glycoproteins, we found that IL-6 also recognizes high-mannose type glycan involving five or six mannose residues. The significance of this carbohydrate recognition in relation to the IL-6 dependent physiological activity was investigated using HepG2 cells and human peripheral lymphocyte. The addition of high-mannose type glycan or Endo H treatment before IL-6 stimulation of HepG2 cells, diminished fibrinogen production and tyrosine phosphorylation. IL-6 stimulation of human peripheral lymphocytes also enhanced tyrosine phosphorylation and the Endo H treatment diminished the activity. It seemed that the carbohydrate recognition activity of IL-6 modulates its functions. To identify which glycoprotein is recognized by IL-6, the constituents of IL-6 receptor, i.e. IL-6R and gp130, were immunoprecipitated using HepG2 cell lysates, blotted onto the nitrocellulose membrane and stained with *Galanthus nivalis* agglutinin (GNA) which can specifically recognize high-mannose type glycan. Only the band corresponding to IL-6R was stained with GNA. These results indicate that IL-6 dually recognizes the peptide portion and high-mannose type glycan of IL-6R, sequentially triggers the high-affinity complex formation of IL-6/IL-6R/gp130.

Glycoconjugates in development and development diseases

P253
DIFFERENTIAL GLYCOSYLATION OF A CONSERVED DOMAIN IN MURINE ZP3 AND TRANSGENIC HUMAN ZP3 DERIVED FROM MOUSE EGGS

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Murine sperm initiate fertilisation by binding to a specialised, glycoprotein layer surrounding the egg known as the murine zona pellucida (mZP). This binding is believed to require the interaction of O-glycans on the glycoprotein mZP3 with sperm membrane proteins but the precise interaction remains to be resolved. Using mass spectrometry we found that the majority of mZP O-glycans are linked to mZP3 and are core type 2 structures terminated with sialic acid, LacNAc, LacdiNAc, Galα1-3Gal and the Sd^a antigen. Human ZP3 (huZP3) can substitute for mZP3 in rescue mice to mediate sperm binding and we have shown that both native mZP3 and huZP3 derived from rescue mice express identical O-glycans. O-glycans occupying two highly conserved glycosylation sites (Ser-332 and Ser-334) on mZP3 were previously implicated in sperm-egg binding, however recent analyses have confirmed that neither site is occupied, implying that an alternative O-

glycosylation domain may be operational. The site specificity of O-glycosylation in both native mZP3 and transgenic huZP3 was analysed in this study using nano-LC ES-MS and MS/MS. Two O-glycosylation sites in native mZP3, one at Thr-155 and the other at positions 161-168, are conserved in transgenic huZP3. In native mZP3, Core 2 O-glycans predominate at both sites. In huZP3 the O-glycans associated with Thr-156 (analogous to Thr-155 in mZP3) are exclusively Core 1 sequences whereas Core 2 O-glycans predominate at the other site. This shift in O-glycosylation at Thr-156 does not however affect the fertility or sperm binding phenotype of eggs derived from female huZP3 rescue mice.

P254
THE CARBOHYDRATE LEWIS X DETERMINANT IS FOUND IN THE TI-VAMP COMPARTMENT OF NT2N NEURONS

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The expression of Lewis X (LeX) carbohydrate determinant is spatially and temporally regulated in the developing central nervous system (CNS) and is thought to play a role in neuronal development in the CNS. α3 Fucosyltransferases (FUTs) catalyze the addition of fucose to type II based oligosaccharide chains, the final step in the biosynthesis of LeX. The NTera-2/cl.D1 (NT2) human embryonic carcinoma cell line generates post-mitotic neurons (NT2N) in response to retinoic acid (RA). In this work, this unique model of human neuronal differentiation was used to investigate the expression of LeX determinant during neurogenesis.
Immunofluorescence microscopy studies in undifferentiated ($NT2^-$) and NT2N cells showed that LeX was expressed in NT2N neurons contrary to $NT2^-$ cells, being detected with the anti-LeX antibodies L5 and SH1 but not with anti-SSEA-1.
FUT activity assays with a panel of acceptors were performed during RA induced differentiation. The acceptor substrate specificity observed, high activity towards LacNAc and H type II, suggested the presence of FUT4 or FUT9. Transcript analysis along differentiation by RT-PCR confirmed FUT9 as the probable FUT responsible for the synthesis of LeX in NT2N neurons.
Immunofluorescence microscopy studies in NT2N neurons with markers of the endocytic and exocytic pathways showed that LeX was restricted to the membrane-trafficking pathway mediated by the SNARE TI-VAMP, known to be involved in neurite outgrowth. These results suggest that the LeX determinant or its carrier molecules may play a role in neuritogenesis.

P255
AN ULTRASTRUCTURAL AND IMMUNOHISTOCHEMICAL INVESTIGATION OF BOVINE CORNEAL STROMA AS A FUNCTION OF DEPTH.

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Introduction Transparency of the corneal stroma requires the uniformity of collagen fibril diameters and interfibrillar distances. These parameters appear to be regulated by proteoglycans and matrix proteins, although the molecular mechanisms are not understood. This study analysed proteoglycan and matrix protein expression in the anterior, mid and posterior regions of the stroma and correlated these data with collagen fibril organisation.
Methods: A fresh bovine cornea was cryosectioned into 100μm sections in an anterior to posterior direction. Expression of proteoglycans and dermatopontin was determined by SDS-PAGE and Western blot analysis. Collagen fibril distribution was studied using transmission electron microscopy and the datasets analysed to determine fibrillar volume, shape factor, fibril diameter and spacing.
Results: There was elevated expression of decorin, keratocan, dermatopontin and keratan sulphate in the mid to the posterior region of the stroma. Decorin also appeared to increase in molecular size towards the posterior stroma. There are significant differences in lamellar organisation in the posterior stroma compared to the central and anterior regions; the collagen fibrils have a smaller diameter, a pronounced increase in interfibrillar spacing and a reduced fibril volume fraction.
Conclusions: These results suggest that different mechanisms may be operating to maintain stromal architecture in the anterior, mid and posterior regions of the cornea. Alterations in proteoglycan expression within these regions may be responsible for these differences.

P256
GALECTIN-3 LIGANDS IN HEALTHY INDIVIDUALS AND CANCERPATIENTS

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Galectins, a family of β-galactoside binding proteins, have currently fourteen members known in mammals. Their precise function is not known but they have strongly been implicated as regulators of immune and inflammatory responses as well as actors in cancer progression. Although all galectins bind β-galactose residues, they have greater affinity for more complex saccharides and high selectivity towards natural glycoconjugate ligands. Cancer cells are known to have an altered expression of β-galactoside containing glycoproteins that in turn are potential galectin ligands. To better understand the function of galectins we have embarked on identifying the natural ligands of galectin-3. Ligands were isolated by affinity chromatography on immobilized galectin and then identified using proteomic techniques (1d and 2d SDS-PAGE, in-gel digestion and mass spectrometry). Application of this strategy to human serum demonstrated its power over previously used techniques based on guessing and confirming by antibodies. The major galectin-3 ligands in serum were α-2-macroglobulin, complement factor H, transferrin, haptoglobin, plasma protease c1 inhibitor and hemopexin. Many of the ligands are included in the acute phase response that is elicited both by inflammation and cancer. This provides intriguing suggestions on possible galectin-3 functions.

P257

POLYMORPHISMS IN THE α-L-IDURONIDASE GENE IS ASSOCIATED WITH PROGRESSION RATE IN IGA NEPHROPATHY

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Background. IgA nephropathy (IgAN), the commonest of chronic glomerulonephritis, occurs sporadically in unrelated individuals. Its rate of progression towards end stage renal failure (ESRF) if extremely variable and only in part explained. Glomerular IgA deposition and abnormal renal response leading to ESRF are two different events, both necessary to lose renal function. As the integrity of the glicosaminglicanes (GAGs) coating the glomerular structures is crucial for the permeselectivity to proteins, we asked if a gain of function of α-L-iduronidase (IDUA), that could reduce GAGs and negative charges density, is associated to faster progression rate of IgAN.

Methods. IDUA locus was screened with multiallelic markers using DISMULT in 317 individuals with biopsy proven IgAN and variable progression rate. IDUA activity was studied in human skin fibroblasts as RT-PCR, and as Vmax and Km of the enzyme with its specific substrate.

Results. "Suggestive" linkage with max LOD score of 1.964 for the marker mapping closest to the IDUA locus. Carriers of 2 CTG alleles of the Leu118Leu (TTG → CTG) polymorphism had faster progression rate ($p>0.0001$). The IDUA mRNA and Vmax were similar in human skin fibroblasts homozygous for the TTG and CTG genotype, but the Km of the CTC homozygotes was significantly lower ($p<0.005$).

Conclusions. IDUA produced by individuals homozygous for the CTG mutation displays larger affinity for its substrate, which may have pathogenetic relevance as it may contribute to reduce the GAGs concentration on the glomerular surface and hence the number of negative charges.

P258

FUNCTIONAL ANALYSIS OF THE GENES REQUIRED FOR SULFATION OF GLYCOCONJUGATES IN C. ELEGANS

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Sulfation of carbohydrates is critical for many biological processes. This common chemical modification requires four steps; i) sulfate ion uptake in the cells by sulfate transporters, ii) conversion into the "active" high-energy form 3' phosphoadenosine-5' phosphosulfate (PAPS) by PAPS synthase, iii) translocation of PAPS from cytosol into Golgi lumen by PAPS transporter, iv) transfer of sulfate from PAPS to glycoconjugates by a multitude sulfotransferases.

The nematode *Caenorhabditis elegans*, the most potent animal model for studying functions of carbohydrates, shares a gene set required for sulfation of glycoconjugates with mammals; except for some cases, *C. elegans* does not have gene redundancy of sulfation related genes. To better understand the biological significance of this modification, we accessed functions of the genes involved in sulfation of glycoconjugates by RNA mediated interference (RNAi) technique and by isolating deletion mutants of the genes in *C. elegans*. Although worms depleted of most kinds of sulfotransferases by RNAi did not show severe defects, inhibition of the steps leading up to PAPS transport showed embryonic and larval lethality with morphological defects, suggesting that sulfation is essential for viability and development in *C. elegans*. We also analyzed their abnormality in detail using with several tissue-specific markers and four-dimensional microscopy.

The comparisons of RNAi phenotypes of sulfotransferases, PAPS synthase and transporters genes indicate the possibility that different sulfotransferases are functionally redundant in *C. elegans*.

P259
CONGENITAL MUSCULAR DYSTROPHY: WHERE SUGARS ARE NOT SO SWEET

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Recent years have seen a significant increase in the number of glycosylation associated diseases discovered. One such area is Congenital Muscular Dystrophy, where a number of genes which are either known or thought to be involved in glycan formation have been found to be mutated in some forms of the disease. These include Fukutin Related Protein (FKRP) and LARGE which are both putative glycosyltransferases and the bi-functional GNE which is an epimerase/kinase enzyme, crucial for the formation of sialic acid. All are thought to be involved in the glycosylation of dystroglycan, and it is the glycans on dystroglycan which bind to proteins such as Laminin in the extra-cellular matrix to provide support for the muscle cells. Therefore alterations in the glycan profile may lead to a breakdown of this connectivity which leads to the dystrophic symptoms. We have developed methods to screen the N- and O-glycosylation patterns of cell lines and tissues from mouse models using the powerful method of mass spectrometry (MS). Enzymatically and chemically released glycans are initially screened using Matrix Assisted Laser Desorption Ionisation (MALDI) MS and the putative structures confirmed using Electrospray (ES) MS/MS. Through the comparison of control and gene altered glycosylation patterns of cell lines and tissues from mouse models further biological evidence to support the specific gene functions can be elucidated.

P260
IDENTIFICATION AND FUNCTIONS OF CHONDROITIN SULFATE PROTEOGLYCANS IN THE MILIEU OF NEURAL STEM CELLS

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The behavior of cells is generally considered to be regulated by environmental factors but the molecules in the milieu of neural stem cells have been little studied. We found by immunohistochemistry that chondroitin sulfate (CS) existed in the surroundings of nestin-positive cells, or neural stem/progenitor cells, in the rat ventricular zone of the telencephalon at embryonic day 14 (E14). Brain-specific chondroitin sulfate proteoglycans (CSPGs) including neurocan, phosphacan/receptor-type protein tyrosine phosphatase β, and neuroglycan C were detected in the ventricular zone. Neurospheres formed by cells from the fetal telencephalon also expressed these CSPGs and NG2 proteoglycan. To examine the structural features and functions of CS polysaccharides in the milieu of neural stem cells, we isolated and purified CS from E14 telencephalons. The CS preparation consisted of two fractions differing in size and extent of sulfation; small CS polysaccharides with low sulfation and large CS polysaccharides with high sulfation. Interestingly, both CS polysaccharides, as well as commercial preparations of dermatan sulfate CS-B and an E-type of highly sulfated CS, promoted the fibroblast growth factor-2-mediated proliferation of neural stem/progenitor cells. None of these CS preparations promoted the epidermal growth factor-mediated neural stem cell proliferation. These results suggest that these CSPGs are involved in the proliferation of neural stem cells as a group of cell microenvironmental factors.

P261
GLYCOSAMINOGLYCANS PLAY IMPORTANT ROLES IN *CAENORHABDITIS ELEGANS*

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Although glycosaminoglycans (GAGs) are widely distributed in the animal kingdom, their functions are not known well. To investigate the biologic functions of GAGs with a model

organism *Caenorhabditis elegans*, we searched the nematode genome for orthologs of human GAG-related genes, and found many genes. We first depleted proteins involved in GAG chain synthesis using RNA mediated interference (RNAi) and deletion mutagenesis (TMP/UV deletion mutant isolation). RNAi of genes involved in GAG chain linkage region synthesis (*sqv-2*, *sqv-3*, *sqv-8*) resulted in early embryonic lethal phenotypes (Emb). RNAi of xylosyltransferase (*sqv-6*) revealed weak phenotype (Emb) unexpectedly. Besides previously described abnormal cell division phenotypes found in gene knockout experiments of chondroitin synthase *ChSy* (*sqv-5*) and chondroitin polymerizing factor (*PAR2.4*: *pfc-1*), deletion mutants or RNAi of genes involved in chondroitin and heparan sulfate synthesis **(***ChSy*, *pfc-1*, *rib-2* and *rib-1***)** showed various new phenotypes including abnormal cellular compartment formation, late embryonic lethality with increased apoptosis, abnormal morphogenesis, egg laying defects and abnormal neuronal wiring. According to these results, we concluded that GAGs are indispensable carbohydrates in development and morphogenesis in *C. elegans*.

P262
CROSS-REACTIVITY STUDIES OF $rMOG_{ED}$ WITH THE SYNTHETIC PUTATIVE AUTOANTIGEN CSF114(Glc) IN MULTIPLE SCLEROSIS PATIENTS' SERA

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There are a number of situations in which aberrant glycosylations have been associated with autoimmune processes. Therefore we compared antibody recognition in Multiple Sclerosis (MS) patients' sera by rMOG (as a putative autoantigen) or by [N^{31}(Glc)]*h*MOG(30-50) and CSF114(Glc) [1-4], as possible molecular mimotopes, to study the Ab response in solid-phase ELISA.
Over-expression in *E. coli* is straightforward and has the potential to produce large quantities of recombinant proteins, even as inclusion bodies. Since proteins expressed as inclusion bodies are usually in the unfolded state, efficient refolding procedure is required in order to perform cross-reactivity studies that will help elucidate any possible connection between the glycopeptide CSF114(Glc) (possible mimotope) and recombinant MOG as *in situ* putative autoantigen in MS.
On the other hand, an aberrant glucosylation on Asn residues of myelin proteins may create neoantigens triggering an autoimmune response in which anti-CSF114(Glc) antibodies are biomarkers of disease activity. The control of glycosylation of proteins in prokaryotic cells can be achieved by semi-synthetic methods. In fact, a non-natural amino acid with an alkyne moiety can be introduced in recombinant proteins in order to get a suitable substrate for Huisgen cycloaddition with an azido-sugar. Met is one of the most suitable amino acid for replacement studies in recombinant proteins and it is an attractive target for bioconjugates engineering. We tried to incorporate, after a site directed mutagenesis, 2-amino-5-hexynoic acid (homopropagylglycine), a Met analogue, in position 31of rMOG, native site of glycosylation, following the Selective Pressure Incorporation method.

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P263
INTERACTION OF MIDKINE WITH HEPARAN SULFATE PROTEOGLYCANS IS INVOLVED IN GROWTH AND SURVIVAL OF NEURAL PROGENITOR CELLS

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Midkine is a heparin-binding growth factor and is expressed in embryonic tissues including the neuroectoderm. Injection of midkine mRNA into Xenopus or zerbafish embryos results in promotion of neurogenesis. This investigation was performed to reveal the action mechanism of midkine in neurogenesis. Neurospheres, which mainly consist of neural stem cells, were established from 14-day mouse embryos. Neurospheres were cultured on dishes with or without dissociation by trypsin. The resulting cells were differentiated cells and nestin-positive neural progenitor cells, which were neural stem cells and their daughter cells on the way of differentiation. We found that midkine was expressed in neural progenitor cells. Furthermore, the number of neurospheres formed from the midkine-deficient embryonic brain was significantly less than that from the wild-type embryonic brain. On substratum coated with poly-L-lysine, neural progenitor cells from the deficient brain spread poorly and grew and survived less than the cells from the wild-type brain. Differentiation to neurons was not affected by the absence of midkine. On substratum coated with midkine, neural progenitor cells from the deficient brain spread, grew and survived similarly as the cells from the wild-type brain. Heparitinase-digestion of dissociated neurosphere cells resulted in poor differentiation, growth and

or survival of neural progenitor cells. Condroitinase digestion gave no effects. These results indicate that midkine promotes neurogenesis most probably by enhancing growth and survival of neural progenitor cells, and that interaction with heparan sulfate proteoglycans is important for the process.

P264
IGNT IS THE PRINCIPAL ENZYME IN POLY-*N*-ACETYLLACTOSAMINE BRANCHING IN ES CELLS AND ITS LOSS SUPPRESSES 4C9, A LEWIS X ANTIGEN

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Branched poly-*N*-acetyllatosamines are good scaffolds of carbohydrate epitopes and increases the affinity of epitopes such as selectin ligands and developmentally-regulated antigens. Multipotential cells of early embryos express embryoglycan, which is a fucosylated and branched poly-*N*-acetyllactosamine of high molecular weight. To understand the mechanism of the formation of embryoglycan and its biological function, we produced embryonic stem (ES) cells from mice deficient in the IGnT gene, and studied their properties. These cells expressed typical markers of ES cells, such as Oct3/4, Sox-2 and Rex-1. However, the amount of fucose-labeled embryoglycan sharply decreased in the ES cells. Galactose-labeled high molecular weight glycan also decreased in amounts and became more susceptible to digestion with endo-β-galactosidase; the result is consistent with the decrease in poly-*N*-acetyllactosamine branching. Surprisingly, these cells lost 4C9, a Lewis X antigen, but continued to express SSEA-1 Thus, IGnT is the principal enzyme involved in poly-*N*-acetyllactosamine branching in ES cells. We are currently analyzing possible changes in activities of these cells. Furthermore, the present result clearly showed the difference between 4C9 and SSEA-1. Although both are Lewis X antigen, we previously noticed more restricted expression of 4C9, and the antigen has been widely used as a marker of primordial germ cells. Together with the loss of the antigen from the cerebellum of IGnT deficient mice, it is established that 4C9 requires high density of Lewis X epitope, which is realized by poly-*N*-acetyllactosamine branching. The clear difference between 4C9 and SSEA-1 opens broad application of anti-4C9 antibody.

P265
LEWIS X-OLIGOSACCHARIDES APPEARED AT SEGMENTATION PERIOD OF ZEBRAFISH EMBRYO

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The Lewis x epitope is known as stage specific embryonic antigen-1. We previously reported the isolation of the gene of zebrafish α(1,3)fucosyltransferase (zFT1) capable to synthesize the Lewis x structure[1)]. The gene was expressed specifically in segmentation period. Meanwhile, we also reported the complex type *N*-glycans significantly appeared from the segmentation period of zebrafish embryo[2)].
In the present study, we have tried to detect the enzyme products of zFT1 in the embryo. Oligosaccharides were isolated from the zebrafish embryos followed by pyridylamination. The PA-oligosaccharides that were α1-3/4fucosidase sensitive and expressed time-dependently at 18 h after fertilization were picked up as candidates for the *in vivo* products synthesized by zFT1. The structures of these oligosaccharides were determined to be a biantennary complex-type with a couple of Lewis x structures. Remarkably, majority of these oligosaccharides was detected as free form. Furthermore, the endo-β-*N*-acetylglucosaminidase activity was detected in the 18 h embryo. These results suggest that the appearance of oligosaccharides synthesized by zFT1 occurred in the embryo at segmentation period as a free form due to liberation from glycoproteins by digestion with endo-β-*N*-acetylglucosaminidase and/or glycoamidase.
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P266
ANALYZING GLYCOME-RELATED GENE EXPRESSION WITH TWO DIMENSIONAL IN-GEL ELECTROPHORESIS (2D-DIGE)

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We are currently performing systematic knocking out of glycome-related genes in the nematode *Caenorhabditis elegans*. Using this model organism, large quantity of wild type and gene-knocked out worms can be collected easily from liquid culture, enabling us to do biochemical analysis,

statistical analysis and systematic analysis of proteomes effectively. Worms with same genetic background are ideal samples for proteome analysis of glycome-related genes.
We previously reported that the nematode has only one ortholog of acetyl CoA transporter gene (SLC33 or AT-1), which is essential for O-acetylation of ganglioside and acetylation of various glycoconjugates in higher organisms. We isolated intron deletion mutant (tm246) and exon deletion mutant (tm1317) of this gene by screening TMP/UV deletion mutant library. Mutant worms showed slow growth rate and dumpy morphology with decreased egg number.
In this study, we extracted proteins from wild type and these mutant worms and analyzed proteomes of these animals with two dimensional (2D) electrophoresis. We differentiated more than two thousand spots, and the spot patterns were analyzed with image analysis software. Some gels were also stained with Pro-Q Emerald 488 to detect glycoproteins. Two dimensional differential in-gel electrophoresis (2D-DIGE) technique was also used to compare proteomes between wild type and the mutant worms. About 500 spots showed over 1.5-fold increase in deletion mutant, and about 300 spots showed over 1.5-fold decrease in the mutant. Over one hundred spots were picked up from the gel, and sequenced with LC/MS/MS to identify gene networks involved in development of mutant phenotypes.

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VEROTOXIN TOXICITY COMPLETELY DEPENDS ON GB3 EXPRESSION IN VIVO.

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Globotriaosylceramide (Gb3) has been considered to be a receptor for verotoxins (VT-1, VT–2) from *E. coli* O157, mediating the apoptosis induction. However, its role in the tissue damages with VT1/2 in hemolytic uremic syndrome (HUS) has not been clearly demonstrated. To investigate the biological functions of Gb3 and the role as a verotoxin receptor in vivo, we generated α1,4-galactosyltransferase (α1,4Gal-T) gene knock out (KO) mouse by gene targeting, since α1,4Gal-T is a key enzyme to synthesize Gb3. The KO mice completely lacked Gb3 synthase activity and Gb3 or its derivatives (globo-series glycolipids) in tissues, convincing the generation of Gb3 null mutant mice. To test sensitivity to VT-2, various doses of VT-2 were injected into the wild type and the null mice. Surprisingly, KO mice survived without abnormal behaviors even when 100 times amount of the lethal dose of VT-2 in the wild type was administered. Pathological analysis revealed that VT-2-treated KO mice showed no changes in brain, kidney and liver, where congestion and vascular damages were observed in the wild type mice injected with the same dose of VT-2. Immunohistochemistry disclosed Gb3 expression in the capillary endothelium in these damaged tissues, suggesting that vascular damages were caused directly by VT-2, and finally resulting in the lethal effects. These results indicated that VT toxicity completely depends on the expression of Gb3 or its derivatives in vivo, and that the inhibition of Gb3-VTs interaction should be the most effective approach for HUS.

P268
CHEMICAL AND GENETIC STUDIES OF THE O-ANTIGEN OF *ESCHERICHIA COLI* O148, THE PUTATIVE ANCESTOR OF *SHIGELLA DYSENTERIAE* TYPE 1

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Shigella are thought to originate from Escherichia coli in the period of enlargement of their econiche. They are classified by lipopolysaccharide O-chains called O-antigens. O-antigen gene clusters in the genome are identical or similar in many *Shigella* and *E. coli* strains, including *E. coli* O148 (EcO148) and *Shigella dysenteriae* type 1 (Sd1), the cause of the most severe form of shigellosis. The Sd1 O-antigen structure is known. A peculiar feature of its biosynthesis is location of the galactosyl transferase-encoding gene on a plasmid. To shed light on the origin of Sd1 we studied the EcO148 O-antigen.
Using sugar analysis and NMR spectroscopy, the EcO148 O-antigen structure was found to differ from that of Sd1 in replacement of D-galactose with D-glucose only. The O-antigen gene clusters of EcO148 and Sd1 were found to be almost identical except for that *orf9*, the putative glucosyl transferase-encoding gene in EcO148, is interrupted by deletion in Sd1. An EcO148 mutant was constructed by deletion of *orf9* and cloning of the galactosyl transferase-encoding gene on the Sd1 plasmid. The mutant had the same O-antigen structure as Sd1 and, in contrast to the parent strain, cross-reacted with antiserum against Sd1.
These data suggested that EcO148 is the ancestor of Sd1 and the evolution was accompanied by the O-antigen alteration caused by invasion of a plasmid with a galactosyl transferase-

encoding gene and inactivation of the glucosyl transferase-encoding gene in the genome.
This work was supported by RFBR grants 03-04-39020 and 05-04-48992, grant NSh-1557.2003.3 and NSFC General Program 30270029.

P269
N-ACETYLGLUCOSAMINYLTRANSFERASE I-NULL DROSOPHILA MELANOGASTER IS UNABLE TO COMPETE FOR SURVIVAL IN THE PRESENCE OF WILD TYPE FLIES

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Introduction. UDP-GlcNAc:αlpha-3-D-mannoside βeta-1,2-*N*-acetylglucosaminyltransferase I (GnT I) controls the synthesis of hybrid, complex and paucimannose *N*-glycans. *Drosophila melanogaster* makes paucimannose but little or no hybrid nor complex *N*-glycans. The single GnT I gene in flies has been cloned and expressed (Sarkar M, Schachter H. Biol Chem 2001, 382:209-217).
Methods. GnT I-null *D.melanogaster* lines were obtained by imprecise excision of a P-element located 546 bp upstream of the start codon; a 1301 bp deletion was produced downstream of the P-element. No flanking genes were disrupted.
Results. GnT I -/- adults were recovered only when animals were removed from the vial at the larval stage and allowed to develop together with a limited number of mutant larvae. Mutant embryos eclosed normally but had a significantly reduced life span (98% dead within 15 days; 80 days for heterozygotes). GnT I -/- adults are viable with a normal external morphology. Locomotor activity (open grid method) showed that -/- flies are significantly more sluggish than wild type flies. No eggs were obtained on attempts to mate mutant males and females. Extracts of GnT I -/- flies showed no GnT I activity. Mass spectrometric analysis of these extracts showed dramatic changes in *N*-glycans compatible with GnT I lack.
Conclusions. The data indicate that GnT I-dependent *N*-glycans are required for normal development of the nervous system of the fly.
Support by the Canadian Institutes of Health Research (CIHR) and the Canadian Protein Engineering Network Centre of Excellence (PENCE).

P270
ESTROGEN INDUCED CHOLESTASIS RESULTS IN DRAMATIC INCREASE OF B-SERIES GANGLIOSIDES IN RAT LIVER

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Introduction: In estrogen induced cholestasis in rat a decrease of membrane fluidity was observed and ascribed to increase of cholesterol ester and sphingomyelin contents. However, glycosphingolipids have not been investigated so far.
Experimental: Ganglioside composition was investigated in normal and cholestatic rat liver. Cholestasis was induced in Wistar rats (n=6) by 17α-ethinylestradiol (EE) (5 mg/kg b.wt.) administered subcutaneously for 18 days and results were compared with untreated animals (n=6). Cholestasis was monitored by serum bile acids and bilirubin. Isolated gangliosides were separated by TLC detected with resorcinol-HCl reagent and evaluated by densitometry.
Results: As compared to controls EE administration resulted in severe cholestasis as indicated by serum bile acids (39.0±27.2 vs. 589.4±203.6 μM/L, p=0.002) and bilirubin (2.1±0.3 vs. 26.0±18.6 μM/L, p=0.002). In the liver tissue total lipid sialic acid content of control and cholestatic animals was 44.3±15.2 and 79.1±9.0 nmol/g liver tissue ($p<0.01$). Control rats had the predominance of gangliosides of a-series of biosynthesis, while in EE-treated rats a significant increase of ganglioside GD1a (3.6±1.0 vs. 11.8±3.0 nmoles/g, p=0.001) and b-series gangliosides GD3 (0.08±0.03 vs. 2.0±1.2 nmoles/g, p=0.002), GD1b (0.1±0.06 vs. 5.4±1.6 nmoles/g, p=0.002) and GT1b (0.06±0.03 vs. 6.4±2.6 nmoles/g, p=0.002) was found.
Conclusion: EE-induced cholestasis leads to dramatic increase of b-series gangliosides in rat liver presumably due to up-regulation of their biosynthesis. As the majority of gangliosides is concentrated in cell membranes, our findings suggest that b-series gangliosides might contribute to cytoprotective resistance of hepatocytes against cholestasis.

P271
N-GLYCOSYLATION IN GASTROPODS

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The knowledge of the glycosylation patterns of molluscs may be an important issue in the near future in order to find new expression systems valuable for creating specific glycosylations, or looking for possible inhibition targets to reduce the surviving capacities of gastropods which are kinds of pests in some areas or hosts for pathogens. Here we present

the analysis of the N-glycosylation patterns of various land and water snails.
SDS-PAGE of the purified proteins followed by blots with specific lectin or antibody detection and analysis of the fluorescent labelled N-glycans by MALDI-TOF mass spectrometry and 2-dimensional HPLC (size and hydrophobicity) before and after digestion with specific exoglycosidases was carried out. In addition the monosaccharide composition was determined by different HPLC methods and further structural information was gained by methylation analysis followed by gas-chromatography/mass spectrometry.
The snails show an enormous potential in generating a large set of structural elements of eukaryotic N-glycosylation: they sialylate, they carry α1,6-linked fucose to the inner GlcNAc as it is common in most animals, they carry α1,3-linked fucose as it is characteristic for plants and can be also seen in some insects, nematodes and trematodes, they contain β1,2-linked xylose as found in plants and trematodes, and they are able to methylate terminal sugars (mannose and galactose) as found in nematodes and trematodes. Thus they combine structural features from mammals, plants, insects, nematodes and trematodes. This is the first known complete system where it is possible to investigate the regulation of N-glycan modification in its fullest variety.

P272
SCREENING FOR MUTATIONS IN PHOSPHOMANNOMUTASE 2 (PMM2) GENE

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Congenital disorder of glycosylation (CDG) Ia (MIM≠212065) is an autosomal recessive multi-organ disease characterized by severe dysfunction of central and peripheral nervous system. It is caused by a defective N-linked glycosylation due to phosphomannomutase (PMM) deficiency as a consequence of mutations in PMM2 gene. More than 85 different mutations in the PMM2 gene known so far are widespread over the gene consisting of 8 exons. The most frequent ones are R141H and F119L, both caused by a single base mutation in exon 5 of PMM2 gene, 422G>A and 357C>A, respectively. We have recently undertaken a comprehensive project with a purpose to determine the frequency of these and some other mutations and polymorphisms in genes related to CDGs in Croatian population. Until now no patient with CDG was detected in Croatian population. Here we present the results of screening for mutations in the exon 5 and parts of intervening sequences IVS4 and IVS5 of PMM2 gene in Croatian population. The study encompassed 104 unrelated individuals. Screening was performed by PCR-SSCP analysis (6% polyacrilamide electrophoresis, 15 °C). 10 fragments that showed aberrant patterns were additionally sequenced on ABI Prism 310 Genetic Analyzer. R141H and F119L mutations were not found in the analyzed group. However, we detected four homozygotes (IVS5+19T/T) and six heterozygotes (IVS5+19T/C) for intragenic single nucleotide polymorphism (SNP) IVS5+19T/C, while all 10 individuals were homozygous for SNP IVS5+22T/T. One of the heterozygotes for IVS5+19T/C was also a heterozygote for deletion of 3bp (ATG) on the position -58 in intron 4 (IVS4-58delATG).

P273
THE ROLE OF PLASMA MEMBRANE TYPE SIALIDASE GENE NEU3 AND GM3 SYNTHASE GENE GM3S ON THE PATHOGENESIS OF TYPE 2 DIABETES MELLITUS IN JAPANESE.

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It has been previously reported that gangliosides and sialic acids play an important role in both type1 and type 2 diabetes. We generated mice overexpressing plasma membrane-associated sialidase (Neu3) specific for gangliosides (J Biol Chem, 2003). The mice developed severe insulin-resistant phenotype of diabetes. In addition, the mice lacking GM3 synthase (SIAT 9) were reported to be protected from high fat diet-induced insulin resistance. This study was designed to investigate the possible contribution of single nucleotide polymorphisms (SNPs) in NEU3 and SIAT9 to type 2 diabetes development.
We screened SNPs in 345 Japanese diabetic patients and 387 controls. We identified two SNP in the promoter region (C/T) and exon 3 (A/G) in Neu3 gene. The allele frequencies of the SNP (C/T) were different between diabetic and control group ($p<0.001$). The values of body mass index, plasma insulin at fasting state and HOMA(R) of subjects with normal glucose tolerance were higher in T/T genotype than in C/C genotype in the SNP.
The frequencies of haplotype-C of T-allele in -32 promoter region and G-allele in exon3 in SIAT9 were different between diabetic and control group ($p<0.01$). The subjects with haplotype-C had lower sum of insulin levels during 75g oral glucose tolerance test ($p<0.001$), insulinogenic index ($p<0.005$) and lower HOMA(R) ($p<0.05$) than those with other haplotypes.
From these results, it may be concluded that Neu3 and SIAT 9 genes may play an important role in the pathogenesis of diabetes mellitus. This is the first report showing the Neu3 and SIAT 9 gene polymorphism to be associated with the development of type 2 diabetes in Japanese subjects.

P274
AGING-ASSOCIATED CHANGES IN BONE AND PROTEOGLYCAN

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Objective: We previously found that glycosaminoglycans (GAGs) were liberated from proteoglycans (PGs) by successive digestion with cellulase from Aspergillus niger having endo-β-xylosidase activity. This present study investigated GAG changes from aging using bone of 10-week-olds female rats and those aged 33 weaks (i.e. with less ability to become pregnant).
Methods: PGs were extracted from the right rear thighbones. GAGs were liberated from PGs by digestion with actinase and cellulase. GAGs were fluorescence-labeled with 2-aminopyridine. PA-GAGs were separated and analyzed by HPLC (anion-exchange chromatography). The left rear thighbones were analyzed by bone tests (DXA, μCT, bone strength test).
Results: Chondroitin sulfate/dermatan sulphate (ChS/DS) and heparan sulfate were separated by HPLC. The ratio of each separated fraction was calculated. No differences were observed in bone density or strength. But the amount of GAGs of ChS/DS was 8.3 nmol/g (52.5%) in the 10-week olds and 2.2 nmol/g (25.4%) in the 33-week olds. The 10-week olds rats also had a higher hyaluronic acid quantity.
Conclusion: These results suggest that qualitative and quantitative changes of GAGs may be caused by aging.

P275
LIPOPOLYSACCHARIDE OF *COXIELLA BURNETII*. STRUCTURAL MOTIFS INVOLVED IN THE IMMUNOBIOLOGY OF Q FEVER

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Coxiella burnetii is the etiological agent of Q fever, a zoonosis that is endemic throughout the world. The disease can occur as an acute disabling illness or develop towards a chronic form. Lipopolysaccharide (LPS) is a major determinant of virulence expression and infection of the bacterium. Its composition and structure determine to a great extent interactions between the microorganism and host including pathogenicity and immunogenicty of the agent. Thus, the structure/function relationship studies are of potential interest.
From virulent *C. burnetii* phase I cells an LPS I was isolated by a hot phenol/water method. It was lipid A deprived and separated by HPLC. The isolated polysaccharide fractions were analyzed for their chemical composition and structure by methylation analysis in combination with GC-, MALDI-ToF- and ESI-MS techniques. The selected, structurally characterized polysaccharide antigens were assayed in ELISA with anti-*C. burnetii* phase I and II rabbit sera and with patient's sera.
We have found that LPS I contains chemically distinct populations of the O-polysaccharide chains differing in their antigenic reactivities. The chains contain a variable amount of two unusual sugars virenose (Vir) and dihydrohydroxystreptose (Strep) that are unevenly distributed along them in terminal positions. The polymer backbone is predominatly composed of GlcNAc residues. A remarkable decrease in the serological activity of O-specific chains with anti-*C. burnetii* phase I and II sera was observed when Vir and Strep were selectively removed from them. These and other data indicate that both sugars are involved in the immunobiology of Q fever.

P276
INVESTIGATIONS ON THE CHEMICAL COMPOSITION AND STRUCTURE OF A LIPOPOLYSACCHARIDE FROM *RICKETTSIA TYPHI*, A RE-EMERGING INFECTIOUS AGENT

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Rickettsia typhi belongs to the typhus group rickettsia and causes endemic (murine) typhus. It is a Gram-negative bacterium maintained in rodents and transmitted to humans by the rat flea *Xenopsylla cheopsis.* Serological diagnosis of endemic typhus is often ambiguous due to the absence of specific and highly sensitive antigens. It has been known that *R. typhi* contains a lipopolysaccharide (LPS) in its cell wall outer membrane that is thought to display a noticeable antigenic activity. However, its immunoreactive epitopes and structural features have been unknown thus far.
From *R. typhi* cells, an LPS was isolated by a conventional hot phenol/water method and run on SDS-PAGE. The polymer was investigated further for its chemical composition and structure by methylation-linkage analysis in combination with GC-, MALDI-ToF- and ESI-MS techniques.
SDS-PAGE of the *R. typhi* LPS gave a "ladder-like" banding pattern typical for LPSs having a regular structure of the O-polysaccharide chain. Glucose linked mainly by 1→4 bonds was found to be the major neutral sugar constituent of the O-specific chain. GlcN represents the main portion of amino

sugars although a noticeable amount of acetylated quinovosamine has also been detected. Analyses of *R. typhi* lipid A showed the presence of glucosamine, phosphate, hexadecanoic, and 3-hydroxy-tetradecanoic acids in the molar ratio of 2:2:1:5, respectively. Its endotoxic potency has to be evaluated.

P277
EXPRESSION OF HEPARANASE IN NEWBORN MOUSE GROWTH PLATES

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Endochondral ossification is tightly regulated by various heparan sulphate (HS)-binding growth factors, such as fibroblast growth factor 2 and Indian hedgehog. However, HS chains immobilized as substituents on cell surface or matrix proteoglycans are limited in the capacity to present these growth factors efficiently to their corresponding signaling receptors. We hypothesize that heparanase, an endo-β-D-glucuronidase, cleaves HS chains at specific sites to yield mobile HS fragments and avails HS-binding growth factors to their targets. Here we aim to map the expression pattern of heparanase in growth plates of both *Hspg2*$^{\Delta 3/\Delta 3}$, a HS-deficient perlecan mouse mutant, and its wildtype littermates by immunohistochemistry. In both mutant and wildtype animals, heparanase protein was detectable in both the pericellular and extracellular matrix of the resting and proliferative zones but not detectable in the pre-hypertrophic zone. In particular, heparanase is localized in the extracellular matrix of the hypertrophic zone where von Kossa staining of calcium deposits were also found. Our results suggest little involvement of HS moieties of perlecan in the distribution of heparanase and HS-mediated maturation process of the growth plate.

P278
HbA1c IN THE 2nd AND 3rd TRIMESTER OF PREGNANCY IN SUSPECTED GESTATIONAL DIABETES MELLITUS

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Introduction:
The Oral Glucose Tolerance test (oGTT) is the standard test for the laboratory diagnosis of Diabetes Mellitus. However, there is no international consensus on oGTT cutoff points in patients with Gestational Diabetes Mellitus (GDM). Significant numbers of pregnant women undergo an oGTT to exclude GDM each year. HbA1c provides an index of glycaemic control over the last 2-3 months. We investigated the utility of HbA1c to predict the outcome of an oGTT in patients screened for GDM.
Methods and patients:
A retrospective audit was conducted in patients (n=79) with suspected GDM. Blood was collected for Fasting Blood Glucose (FBG) and HbA1c. A 75 g glucose load was administered in patients with a FBG &lt8.0mmol/L. A second sample was collected at 2hrs post glucose. HbA1c (DCCT aligned) was analysed by HPLC (Tosoh Biosciences Ltd); coefficient of variation (CV) 2.2% at 5.3mmol/L and 1.6% at 10.1mmol/L.
Results:
The mean fasting and 2hr glucose values were 4.4mmol/L (Σ 0.7) and 6.2mmol/L (Σ 1.7) respectively. The mean HbA1c was 5.1% (Σ1.0). There was a significant correlation between HbA1c and FBG (p=0.0035) and 2hr blood glucose (p&lt0.0001). Using WHO criteria[1], 3 patients had DM and 5 Impaired Tolerance (IGT). Using a cut off point for HbA1c at 5.0% gave 87.5% sensitivity and 64.8% specificity. Positive and Negative Predictive Values were 21.9% and 97.9%.
Conclusion:
Using a HbA1c cut off of 5%, more than 50% of pregnant women can be spared an oGTT. However, this pilot project needs to be validated by a larger population study.

P279
INVESTIGATIONS ON EXPRESSION OF FUCOSYLATED OLIGOSACCHARIDE ANTIGENS IN EPIDIDYMIS

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Epididymis secrets glycoproteins into the lumen, and creates a microenvironment, which is necessary for spermatozoa maturation. Lately, it is found that in human seminal plasma there has very high concentration of free oligosaccharides (0.3-0.4mg/ml) carried Le^X, Le^Y epitopes and assumed them may be important for sperm vitality[1]. But the source and function of the fucosylated oligosaccharides are still unclear. In this study Le^X and Le^Y antibodies were used as probes, by immunohistochemistry, dot blot, immuno-fluorescence, and RT-PCR to identify the expression and localization of the antigens in epididymis and sperm. The results revealed that Le^X and Le^Y were negative in the testis, but turned to strong positive in principle cells of the epithelial cell in epididymal caput, where lumen sperm show positive, too. Except some

apical cells and clear cells, the expressions weakened in corpus and cauda in the mouse. The results were similar with those in human epididymis. The matured human sperm showed Le^X and Le^Y strong positive in the head apical and neck but the tail is negative, which was coincident with the formal Ram PA's work of fucosyltransferases expression in the mouse sperm. Our results indicated Le^X and Le^Y are mainly synthesized and secreted by epithelial cells in caput epididymis and may be coated on the surface of non-matured sperm in the caput lumen, but matured sperm might synthesize them by itself in definite regions; The fucosylated oligosaccharides in seminal plasma might mostly come from epididymis.

Supported by National Natural Science Foundation of China (3017266 to ZM Zhu); 973 Basic Research founding of China G19990559012 and CAS Logic Creative Founding KSCX2-SW-201(to YL Zhang)

Reference

Chalabi S et al (2002) J Biol Chem 277:32562~70

Glycoimmunology and glycoimmunopathology

P280
ROLE OF CARBOHYDRATE MOIETIES OF AN ALLERGENIC GRASS EXTRACT ON THE HUMORAL TH2 RESPONSE IN MICE.

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Grass pollens, such as that from timothy grass (*Phleum pratense, Pp*), represent a major cause of type I allergy, by inducing significant IgE response against the major allergenic proteins represented in the pollen extract. About 20 % of patients allergic to *Pp* have IgE antibodies recognizing carbohydrate-containing epitopes.

The aim of this study was to evaluate the humoral TH2 response against the carbohydrate moieties of a grass allergenic extract, in a mouse model of allergen sensitization.

Balb/c mice were sensitized with *Pp* pollen extract (*PpE*). Serum IgE and IgG levels were monitored by ELISA against *PpE* and several not correlated glycoproteins sharing glycans with some allergens present in *PpE*, such as Horseradish Peroxidase (HRP), Bromelain (BRO), Phospholipase A2 (PLA2) and Ovalbumine (OVA).

Sensitization with *Pp* pollen extract induced high levels of specific IgE and IgG. The induced IgE did not bind HRP, BRO, PLA2 and OVA. On the contrary, we found in the immune sera IgG able to recognize all these glycoproteins, with high reactivity against HRP. IgG binding to HRP was significantly reduced after chemical treatment with sodium periodate, as determined by ELISA, thus confirming that these antibodies are glycan-specific. The only IgG subclass reacting with HRP was the IgG1. Pre-immune Balb/c mouse sera were not able to detect these glycoproteins, indicating that production of antibodies specific for plant N-glycans was induced by immunization with *PpE*.

In a well established mice model of grass pollen sensitization, immunization with *PpE* was able to induce IgG antibodies recognizing epitopes sensitive to periodate oxidation. Moreover, these anti-carbohydrate antibodies seem to recognize crossreactive plant N-glycans, probably represented by core β-(1,2)-xylose, core α-(1,3)-fucose epitopes and mannose.

P281
RABBITS BUT NOT MICE CAN PRODUCE SPECIFIC, HIGH AFFINITY IGG ANTIBODIES AGAINST PLANT N-GLYCANS

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Xylosylated and core α1,3-fucosylated N-glycans from plants are immunogenic and they play a still obscure role in allergy and allergy diagnosis. The significance of such glycans in plant-derived protein pharmaceuticals is currently a matter of intense debate and hence specific reagents for these epitopes are in demand. Rabbit anti plant-glycoprotein sera contain two independent populations, one reacting with a xylose-epitope, and the other with a fucose-epitope. We wanted to generate fucose or xylose-specific - ideally monoclonal - antibodies against plant N-glycans. Therefore, we immunized C57BL/6 mice with plant glycoproteins and tried to obtain hybridoma lines producing useful antibodies. However, we could only obtain monoclonals which were unable to discriminate fucosylated from xylosylated N-glycans and even reacted with the pentasaccharide core $Man_3GlcNAc_2$. Immunization of rabbits on the other hand yielded polyclonal sera but with high specificity towards different glycan structures. Purification of these sera using glyco-modified fibrinBSA conjugates coupled to Affi-Gel 15 provided us with polyclonal sera mono-reactive against either fucosylated or xylosylated N-glycans. Surface plasmon resonance measurements using sensor chips with immobilized glyco-modified transferrins revealed dissociation constants of around $2 \cdot 10^{-9}$ M. This astonishingly high affinity of IgG antibodies towards carbohydrate epitopes has repercussions on our concepetion of the binding strength of anti-glycan IgE antibodies in allergy even though this has not yet been measured.

P282

INTERACTION OF *PSEUDALLESCHERIA BOYDII* WITH THE INNATE IMMUNE SYSTEM: INVOLVEMENT OF MYD88, CD14 AND TOLL-LIKE RECEPTORS (TLRS)

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Pseudallescheria boydii is an emerging opportunistic filamentous fungus that causes local as well as disseminated infections in both immunocompetent and immunocompromised patients. Little is known about the host defence against *P. boydii*. In this study, mice deficient in TLR-associated adaptor protein MyD88, CD14, TLR2 and TLR4, were utilized to investigate the contribution of TLRs and CD14 to host interaction with *P. boydii.* Conidia and a previously characterized α-glucan, extracted from the fungal cell wall, were used to stimulate peritoneal macrophages of mice *in vitro*. Parameters such as TNF-α production and phagocytic indexes were analyzed. Was observed a major role of CD14 and MyD88 in the recognition of both stimuli. Compared with elicited macrophages obtained from wild-type mice, TLR2-/- macrophages did not produce TNF-α, following α-glucan stimulation. Inhibition of conidia phagocytosis by the α-glucan was also observed. These results suggest the involvement of TLR in recognition of the fungus and the α-glucan´s role in the development of the response against *P. boydii.*
Supported by : CAPES, CNPq, FAPERJ.

P283
THE EFFECTS OF MATURATION ON THE GLYCOSYLATION OF DENDRITIC CELLS

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INTRODUCTION. Dendritic cells (DCs) are known as the most potent antigen presenting cells in the body. Immature DCs reside in peripheral tissues and sense for incoming pathogens. Upon pathogen recognition immature DCs migrate via the lymphatics to regional lymph nodes, where they arrive as fully mature non-phagocytic DCs. Here the mature DCs encounter naïve lymphocytes and are able to activate them. This dramatic change in the function of DCs, from pathogen detection to cell adhesion/communication is mediated by an important genetic reprogramming. Protein-carbohydrate and carbohydrate-carbohydrate interactions are recognized as important mediators for cell communication.
EXPERIMENTAL APPROACH. In order to understand the changes that occur during the maturation of DCs with regards to the glycosylation machinery and how they affect DC function, we have studied the differences in glycosylation of immature DCs and mature DCs by analysing the glycans present in the membrane of DCs using plant lectins, and the gene expression profile of the glycosylation-related genes involved in this process.
RESULTS. In this study we show that maturation of DCs results in large changes in the expression of glycosylation-related genes, especially fucosyltransferases (upregulation of FUT-1 and FUT-4), and a galactosyltransferase (downregulation of β4GalT-1). These changes correlated with specific lectin-stainings and suggest that lewis-type antigens might be upregulated upon maturation.
CONCLUSIONS. Maturation of DCs results in large changes in the glycans expressed by these cells. This change is mediated by the modulation of the expression of several glycosyltransferases and might be of importance for the migration, adhesion, and intercellular communication of DCs.

P284
SPECIFIC INTERACTIONS OF HUMAN GALECTINS WITH TRYPANOSOMA CRUZI.
I.- RECOGNITION AND FUNCTIONAL PROCESSING.

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Trypanosoma cruzi is the causative agent of Chagas´disease, affectings more than 17 million people. The parasite it has to move through the extracellular matrix and is able to recognize, attach and enter to a large variety of cells, before it can establish the infection. This can curse with different clinical manifestations with serious damage to heart, digestive tract or nervous system. The causes of this variability are not known yet, although a correlation between clinical manifestations and the parasite genetic background, as one of the two phylogenetic lineages, Tc1 and Tc2 has been achieved. There is evidence implicating glycans exposed on the surface of the parasite in the host-parasite interactions, and presumably the variability observed is related to glycoconjugates on parasite and host cells. In this report we provide evidence of specific and differential binding and recognition of several human recombinant galectins (1,3,4,7 and 8), to the three developmental stages of strains of the two major phylogenetic lineages of Trypanosoma cruzi. The non infective epimastigote form of both lineages showed the less binding of all galectins tested. By contrast, the infective forms, amastigote and metacyclic tripomastigotes showed greater affinity for all tested galectins, with the exception of galectin 1 and 3 which showed low binding to the latter forms. Evidence is shown also that binding of tripomastigotes to host cells (THP-1 and HL60) is enhanced by some exogenous galectins (gal-7, 8) but not by galectin-1 even at high concentrations. Interactions of galectins with tripomastigotes for longer times led to cleavage of galectina-3, 4 and 8, but not galectin-1 or 7.

The cleavage of galectin 4 and 8 is in the linker peptide and it is inhibited by 1,10-ortho-phenanthroline. In the case of galectin-3, the cleavage is in the N-terminal domain at several sites, it is inhibited by 1,10-ortho-phenantroline and $HgCl_2$. Kinetic analysis of cleavage suggests two different proteases involved in this processing. As the modulation of the immunomodulator activities of galectins rely on its oligomerization (di/multivalency), it is proposed that this Trypanosoma cruzi processing could modulate galectin functions.

The financial support of Spanish Science and Industry Ministry through Research Project SAF2002-02444 is acknowledged.

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RECOGNITION OF A CARBOHYDRATE XENOEPITOPE BY THE HUMAN NK PROTEIN NKRP1A

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Introduction. Many immunologically important interactions are mediated by leukocyte recognition of carbohydrates via cell surface receptors. Previously we demonstrated that uncharacterized receptors on human NK cells react with ligands containing the terminal Galα(1,3)Gal epitope. The aim of this work was to isolate and characterise carbohydrate binding proteins from NK cells.
Experimental approaches and Results. Initial PCR analysis suggested the human NK protein NKRP1A (CD161) bound to a pool of carbohydrates, the majority of which contained the terminal Galα(1,3)Gal epitope. This was confirmed by the high level of binding of cells expressing NKRP1A to mouse laminin. Laminin was selected because it contains a large number of N-linked oligosaccharides with the prototypical Galα(1,3)Gal-β(1,4)GlcNAc-R structure, thus potentially increasing the low binding affinity typically observed with monovalent carbohydrate-protein interactions. To investigate the role of the terminal αGal residue laminin was digested with α-galactosidase, revealing the Galβ(1,4) GlcNAc or N-Acetyllactosamine epitope. A greater than 90% reduction in Griffonia simplicifolia lectin (IB4) binding confirmed the epitope was removed. Exposing N-Acetyllactosamine, as shown by Erythrina corallodendron lectin (ECorL) binding, resulted in a significant increase in binding of cells expressing NKRP1A.
Conclusions. We have identified the NK protein NKRP1A as binding to Galα(1,3)Gal. Moreover, binding is increased when the carbohydrate profile is changed to reveal N-Acetyllactosamine. We are currently investigating if these protein-carbohydrate interactions affect NK cell function and downstream cell signalling.

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MOUSE MONOCLONAL ANTI-NOR ANTIBODIES AND THEIR APPLICATION IN DETECTING REACTIVE GLYCOLIPIDS IN PLANTS

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The rare NOR erythrocytes have unique glycosphingolipids containing Galα1-4GalNAcβ1-3Gal- terminal unit that is recognized by natural antibodies present in human and animal sera. To the best of our knowledge, the sequence Galα1-4GalNAc- has only been reported earlier in 1 of 25 oligosaccharide chains identified in the mucin of *Rana ridibunda* (Mourad *et al*., EJB 268,1990, 2001), and as the internal sequence of O-chains of *Proteus vulgaris* 019 (Vinogradov *et al.*, EJB, 180, 95, 1989) and *Proteus mirabilis* 014 (Perepelov *et al*., EJB, 261, 347, 1999). However, the presence of anti-NOR antibodies in both human and animal sera suggests that respective immunogens may be more frequent. To facilitate the detection of NOR-like antigens, monoclonal anti-NOR antibodies were obtained by immunization of mice with Galα1-4GalNAcβ1-3Gal-HSA conjugate. Two closely similar antibodies (nor87 and nor118, both IgG1) with high affinity and specificity for Galα1-4GalNAc were obtained. The antibodies reacted strongly with Galα1-4GalNAcβ1-3Gal and Galα1-4GalNAc, about 400-times less strongly with Galα1-4Gal, and did not react with Galα1-3Gal, Galα1-3GalNAc, Galα1-4GlcNAc, Galα1-2Gal, and Galα1-6Glc. The antibodies also reacted strongly on TLC plates with NOR glycolipids and did not detect any antigens in normal control human erythrocytes. Suspecting that potential NOR-like immunogens may be present in food of plant origin, the glycolipids were extracted from several plants, and fractionated on high performance TLC plates, which were then overlayed with the anti-NOR monoclonal antibody. The distinct glycolipid bands were detected in extracts of seeds of fennel (*Foeniculum vulgare*) and oat (*Avena sativa*), but not in those of wheat (*Triticum*). The extracts of some edible mushrooms were also tested. The anti-NOR antibodies detected neutral glycolipid bands in *Pleurotus cornucopiae* and similarly migrating, but weaker bands in a wild edible fungus of the genus *Boletus.* Interestingly, the pattern of the bands in the seed extracts was different from that found in fungi. These introductory data suggest that glycolipids reacting with anti-NOR antibodies are not uncommon in plant material.

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GALECTIN-3 EXPRESSION IS AFFECTED BY GLUCOCORTICOIDS

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Galectin-3, a beta-galactoside binding lectin, acts as a strong pro-inflammatory signal that modulates cell proliferation and adhesion, chemotaxis, phagocytosis and synthesis of inflammatory mediators. However, data on regulatory mechanisms of galectin-3 expression are still elusive. We have previously shown that transcription factors NF-κB and AP-1 are involved in regulation of galectin-3 expression. Since signaling pathways that regulate the activity of these transcription factors are strongly influenced by glucocorticoids we investigated effects of hydrocortisone and dexamethasone, applied in therapeutic ranges, on the expression of *LGALS3* and galectin-3 in non-differentiated and differentiated cells of monocytic THP-1 cell-line during 72 hours of cultivation. Relative RT-PCR method and GeneScan analysis software were used for assessing galectin-3 mRNA level and chemiluminescent-western blot analysis was used for measuring galectin-3 level. Differentiation of monocytic THP-1 cells into macrophages strongly induced expression of *LGALS3* and galectin-3. In undifferentiated cells both drugs (in all applied concentrations) inhibited expression of *LGALS3* and galectin-3, and inhibitory effect correlated with time of exposure. In differentiated cells, hydrocortisone and dexamethasone inhibited *LGALS3* expression at the beginning, but during further incubation constitutive level of galectin-3 mRNA was reestablished. On the protein level, prolonged exposure to both drugs induced galectin-3 expression. The chosen glucocorticoid drugs affected *LGALS3* and galectin-3 expression, and their effects depended on cell differentiation level, concentration of the applied drug as well as time of exposure. These findings represent important step in the understanding of the effects of glucocorticoids on galectin-3 in monocytes/macrophages, hence on their immunomodulatory activities.

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CHARACTERIZATION OF OLIGOSACCHARIDE EPITOPE RECOGNIZED BY ANTI-MUCIN MONOCLONAL ANTIBODY, RGM21, USING NEOGLYCOCONJUGATE TECHNOLOGY

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A monoclonal antibody (mAb), designated as RGM21, stains histochemically the rat gastric mucosa in site-specific manner, and this mAb is revealed to recognize carbohydrate portion of mucins (1). To determine the epitope structure for this mAb, neoglycolipids synthesized from human milk oligosaccharides were examined by immunostaining on thin-layer plates. RGM21 reacted with lacto *N*-fucopentaose I but none of 2'-fucosyllactose, lacto *N*-tetraose, lacto *N*-neotetrasose, lacto *N*-fucopentaose II or III, or lacto *N*-difucohexaose I or II; thus suggested that RGM21 recognized blood group H trisaccharide structure. Further analysis using synthetic oligosaccharide-conjugated polyacrylamide resins showed that RGM21 recognized H type 1 structure, but not H type 2 or H type 3 structure. Neoglycolipids prepared from the rat gastric mucin oligosaccharide chains were also examined for reactivity with RGM21 by immunostaining after thin-layer chromatography, and the reactive and non-reactive ones were characterized by matrix-assisted laser desorption ionization mass spectrometry. Heptasaccharide having blood group H trisaccharide structure was identified as RGM21-reactive oligosaccharide. The neoglycoconjugate technology using both structure-known and mucin-derived oligosaccharides were powerful tool to examine epitope structure recognized by anti-mucin mAbs.

1. Goso, Y., Ikezawa, T., Kurihara, M., Endo, M., Hotta, K., Ishihara, K. (2003) J. Biochem. (Tokyo) ***133,*** *453-460.*

P289
MYCOBACTERIAL LIPOMANNANS ARE MAJOR IMMUNOMODULATORS

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Phospho-inositol mannosides (PIMs) and their multiglycosylated counterparts, lipomannans (LM) and lipoarabinomannans (LAM), are complex lipoglycans ubiquitously found in the envelopes of all mycobacterial species. They all share a conserved mannosyl-phosphatidyl-*myo*-inositol (MPI), presumably used to insert these structures into the plasma membrane. LM and LAM are further elongated by respectively a mannan domain and a highly ramified arabinomannan domain. LAM of *M. tuberculosis* was known to present a vast array of biological functions including immunomodulation and internalization of the bacillus by macrophages and dendritic cells. These seem partially dependent on LAM species-specific structural features, in particular the nature of capping motifs decorating the non-reducing termini of the arabinosyl side-chains. Because LM was considered as a direct biosynthetic precursor of LAM, very few studies were aimed to decipher its potential biological activities. We have focused our work on the study of structure-functions relationships of lipoglycans isolated from pathogenic atypical mycobacteria including *M. chelonae, M. kansasii* and *M. thermoresistibilis*. From a structural point of view, this work provided new insights into the species-

specificity of lipoglycans by deciphering new sources of structural variability, such as the branching pattern of the mannan domain or presence of various substitutions. From a functional point of view, we have reported that LM exhibited an unexpectedly wide variety of biological functions; some of them antagonizing those exhibited by their corresponding LAM. Among these features, LM appears as a potent proinflammatory cytokine and metalloproteinase-9 (MMP-9)-inducing factor.

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ATTENUATED RECRUITMENT OF PHOSPHATSES BY NATURAL KILLER CELL RECEPTOR, SIGLEC-7

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Siglec-7 (p75/AIRM1) is an inhibitory receptor on human natural killer (NK) cells. We previously characterized sugar-binding specificity of Siglec-7, and found that the lectin preferably binds a unique oligosaccharide structures such as sialyl-alpha2,8-sialyl and galactosyl-beta1,3-[sialyl alpha2,6]-hexosaminyl residues (T. Yamaji et al. *J Biol Chem*, 277, 6324, 2002, *Methods in Enzymol*, 363, 104, 2003). The cytoplasmic domain of Siglec-7 contains two signaling motifs: a membrane-proximal immunoreceptor tyrosine-based inhibitory motif (Ile435-Gln-Tyr-Ala-Pro-Leu440) and a membrane-distal motif (Asn458-Glu-Tyr-Ser-Glu-Ile463). We report here that, upon pervanadate treatment, Siglec-7 recruited the protein tyrosine phosphatases SHP-1 and -2 less efficiently than did other inhibitory receptors such as Siglec-9 and leukocyte-associated Ig-like receptor-1. Alignment of the amino acid sequences of the two Siglecs revealed only three amino acids difference in these motifs. To identify the amino acid(s) critical to recruitment efficiency, we prepared a series of Siglec-7-based mutants in which each of the three amino acids were replaced with the corresponding one of Siglec-9 (I435L, P439S, and N458T mutants). P439S and N458T mutants showed pronounced enhancement of SHP recruitment, but I435L mutant did a little effect. A double mutant (P439S, N458T) or triple mutant (I435L, P439S, N458T) recruited SHPs as much as did Siglec-9, indicating that Pro439 in the proximal motif and Asn458 in the distal motif of Siglec-7 attenuate its ability to recruit phosphatases (T. Yamaji et al. *Glycobiology*, 15, 667, 2005). These amino acids appeared to affect not only phosphatase recruitment but also the subsequent attenuation of Syk phosphorylation, ending up influencing activation of NK cells.

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DE-*N*-ACETYLLACTOTRIAOSYLCERAMIDE AS A NOVEL CATIONIC GLYCOSPHINGOLIPD OF BOVINE BRAIN WHITE MATTER: ISOLATION AND CHARACTERIZATION

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The majority of glycosphingolipids (GSLs) in mammalian cells and tissues are either anionic (having sialic acid or sulfate) or neutral (having no ionic group). Since sphingosine, sphingolipid breakdown products (e.g., lyso-GSLs), and *N*-methylsphingosine were initially implicated as signaling molecules, we have studied cationic lipids and cationic GSLs displaying positive charge through the presence of an unsubstituted amino group. We hereby report results of further studies on cationic GSLs present in bovine brain white matter. A new cationic GSL(NCG), separated by a series of chromatographies with CM-Sephadex and silica gel in chloroform/methanol, was identified unambiguously as de-*N*-acetyllactotriaosylceramide by mass spectrometry and 1H NMR. The overall results indicate a GlcNβ;1–3Galβ;1–4Glcβ;1–1Cer structure. The natural occurrence of this glycolipid in white matter extract was detected by immunostaining of thin-layer chromatography with the monoclonal antibody 5F5, which is directed to de-*N*-acetyllactotriaosylceramide and recognizes the terminal β;-glucosaminyl (GlcNH2) residue, having a free NH2 group. A de-*N*-acetylase capable of hydrolyzing the *N*-acetyl group of Lc3Cer was detected in bovine brain extract using N-[14C]acetyl-labeled Lc3Cer as a substrate. Although the possible role of deNAcLc3Cer as a signaling molecule is unknown at this time, there is a possibility that cationic GSLs may affect organization assembly of GSLs in the membrane. It is of great interest to determine whether this enzyme, specific de-N-acetylase, is activated, and releases deNAcLc3Cer, upon cell stimulation.

P292
IDENTIFICATION OF GUAR GUM ALLERGENS

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Guar gum is derived form a vegetable that grows in India, Cyamopsis tetragonolobus belonging to Leguminosae family as same as soybean (Glycine max). Recent years, guar gum is

used as a thickening agent for food in Japan. It was reported that guar gum caused occupational asthma and immunologic sensitization among employees at a carpet-manufacturing plant. We developed a highly sensitive enzyme immunoassay for soybean trypsin inhibitor, which was one of major allergen in soybean, and attempted to detect immunologically cross-reactive allergen-like substance in some functional food sold in Japan. As a result, we found that cross-reactive soybean trypsin inhibitor-like substance was contained in reagent grade guar gum (Sigma, USA) preparation and some guar gum containing functional food, Fiber Plus and Aosagayu. SDS-PAGE and Western blotting of those preparation using anti-trypsin inhibitor antibody shows broad staining band at high molecular weight region. Guar gum preparation was treated with N-glycosidase A at 37C and pH5.0 overnight, and subjected to SDS-PAGE and Western blotting. As a result, the amount of broad band was decrease and minor bands were appeared at middle molecular weight region. Both of broad and minor band was also stained with glycoprotein staining kit. Cross-reactive soybean trypsin inhibitor-like substance was also detected in guar seed, but could not detected in Fibaron S, which was highly purified and enzymatic digested guar gum preparation. These results suggest that allergens of guar gum are glycoprotein, which belongs to Leguminosae allergen family protein, such as soybean trypsin inhibitor.

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TARGETED IDENTIFICATION OF ANTIGENIC AND DIAGNOSTIC SCHISTOSOME EGG-DERIVED GLYCANS AND GLYCOPROTEINS

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Schistosomes are parasitic trematodes that chronically infect over 200 million people wordwide. Unusual glycoproteins and -lipids, particularly of the schistosome egg stage, induce prominent and characteristic immune responses and pathology in the mammalian host. Western blot analysis of *S. mansoni* soluble egg glycoproteins (SEA) using a monoclonal antibody (mAb 114-4D12) derived from schistosome-infected mice indicated that this mAb predominantly binds to O-glycan epitopes on SEA.
We specifically isolated the 114-4D12 bound glycoproteins by affinity chromatography. Proteomic analysis of the isolated glycoprotein subset yielded several sequences of unknown function, but also two proteins with strong homology to galactosidases/ *N*-acetylgalactosaminidases were found. To identify the 114-4D12 epitope, O-glycans of the captured proteins were released by hydrazinolysis (P. Rudd, Oxford Glycobiology Institute), labelled with 2-aminobenzamide, and characterised by combined MALDI-TOF- and nanoLC-MS/MS based approaches. When the released O-glycans were re-applied to the mAb column only glycans with the terminal Fucα1-2Fucα1-3GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAcβ1- element were retained. Surprisingly, we were able to affinity-isolate free oligosaccharides containing the same fucose-substituted polyHexNAc backbone from the urine of *S. mansoni* infected individuals, as well as from *S. mansoni* egg incubates, indicating that eggs produce also non-conjugated glycans, and that these glycans are present in the circulation of the host.
Interestingly, neoglycoconjugates containing the Fucα1-2Fucα1-3GlcNAc sequence induce innate cytokine responses in monocytes, and schistosomiasis patients generate strong antibody responses to this particular element. Since the Fucα1-2Fucα1-R sequence has so far only been detected in schistosomes we conclude that it forms an attractive diagnostic and potentially therapeutic target.

P294
THE CHARACTERIZATION OF LIGAND BINDING TO ANTIBODIES THAT ARE CROSS-REACTIVE WITH LIPO-OLIGOSACCHARIDE ISOLATED FROM *CAMPYLOBACTER JEJUNI* AND HOST GANGLIOSIDES

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The outer core component of the lipo-oligosaccharide (LOS) in *Campylobacter jejuni* is frequently sialylated, and is capable of eliciting antibodies that are reactive with host gangliosides, leading to an autoimmune response, and the onset of Guillain-Barré syndrome (GBS). We have initiated an investigation into the molecular determinants governing the antigenic response resulting from exposure to GBS-associated *C. jejuni* strains known to display ganglioside mimics. Three monoclonal antibodies were raised in mice against LOS extracted from *C. jejuni*, with reactivity towards GM1a, GD1a and GT1a respectively. BIACORE measurements were used to determine the binding affinity of glycan derivatives corresponding to the three gangliosides, with their corresponding IgGs; the dissociation constant for antigen binding was found to be 10, 20 and 20 μM for GM1a-, GD1a- and GT1a-thiophenyl, respectively. In addition, saturation transfer difference (STD) NMR spectra have been acquired to probe the molecular topology of the ligand-antibody binding surfaces.

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CHARACTERIZATION OF SULFATED OLIGOSACCHARIDE EPITOPE RECOGNIZED BY AN ANTI-MUCIN MONOCLONAL ANTIBODY PGM34

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Mucin, a major component of mucus, is deeply involved in the physiology of gastro-intestinal mucosa. Some monoclonal antibodies (mAbs) developed in our laboratory recognize specific oligosaccharides of mucin secreted from specific type of mucous cells. In this study, we analyzed the epitope structure of a mAb, PGM34, which reacted with a specific acidic oligosaccharide attached to a mucin molecule. PGM34 was established by immunizing a mouse with purified pig gastric mucin, and immunohistochemically reacts with specific mucus cells of rat gastro-intestinal mucosa. The oligosaccharides were obtained from partially purified pig gastric mucins by alkaline borohydride treatment followed by successive treatments with gel-filtration and anion-exchange chromatographies and then by normal phase HPLC using amide column. Competitive ELISA was performed to detect the oligosaccharides reacting with PGM34. The carbohydrate composition of oligosaccharides were analyzed by MALDI-TOF/MS and amino sugar analyses. The chemical structure of two oligosaccharides reacted with PGM34 was analyzed by 400 MHz NMR spectroscopy. The oligosaccharides reacted with PGM34 were characterized as acidic oligosaccharides by an anion exchange chromatography. These oligosaccharides were further purified by two steps of normal phase HPLC. Two highly purified oligosaccharides obtained by HPLC reacted with PGM34 and had a sulfate residue by MALDI-TOF/MS estimation. NMR spectroscopy showed that these two oligosaccharides had a common structure having a sulfate residue, Fucα1-2Galβ1-4GlcNAc(6SO_3H)β-. In conclusion, a mAb PGM34 recognizes a mucin-derived acidic oligosaccharides having a sulfate residue.

P296
GLYCOSYLATION IN *SCHISTOSOMA MANSONI* GLYCOPROTEIN SECRETIONS

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Increasing evidence suggests that carbohydrate moieties from parasitic helminths are crucial determinants in interacting with the host and have an inherent ability to modulate the host immune system. *Schistosoma mansoni* has been one of the most studied parasites. However, structural studies on *S. mansoni* have been based on the whole extracts from the cercaria (infective to man) and egg (responsible for transmission to the snail intermediate host). Yet, little is known about *S. mansoni* secretions. Secretions are the first components encountered by the host immune system. Therefore, we have characterised the N-and O-glycan structures from the *S. mansoni* cercarial and egg secretions to address their biological functions. Mass spectrometric techniques such as (i) matrix assisted laser desorption ionisation (MALDI-MS) (ii) electrospray collisionally activated decomposition (CAD ES-MS/MS) and (ii) gas chromatrography mass spectrometry (GC-MS) have been utilised to facilitate the structural elucidation. Results have revealed several interesting features of the glycan profiles in *S. mansoni* secretions: (i) core xylosylation, (ii) core difucosylation, (iii) the major terminal structure is the Le^x (Galβ1→4(Fucα1→3)GlcNAc) structure, (iv) minor structures such as LacdiNAc (GalNAcβ1→4GlcNAc) and their fucosylated counterparts ((±Fucα1→3)$_{0-2}$GalNAcβ1→4 (Fucα1→3)$_{0-2}$ GlcNAc) were also present. This study has shown that mass spectrometry is a powerful tool in elucidating the glycan structures and it is anticipated that these findings may lead to a better understanding at the molecular level of host-parasite interactions.

P297
HUMAN SALIVARY MUCINS: MG2 (MUC7) GLYCOSYLATION IS CONSISTENT, WHEREAS MG1 (MUC5B) GLYCOSYLATION VARIES EXTENSIVELY BETWEEN HEALTHY INDIVIDUALS

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Introduction: The study aimed to characterize the natural glycosylation variation of the high-molecular-weight glycoprotein fraction MG1, and the low-molecular-weight glycoprotein fraction MG2. These mucin fractions have been shown to bind differentially to bacteria and we hypothesise that this is partly due to their glycosylation. The variation of the glycosylation of mucins could explain the different susceptibility of individuals to various pathogen infections via mucosal surfaces.
Experimental: Salivary mucins from thirty individuals were isolated by gel electrophoresis, blotted to PVDF membrane and visualised with Alcian Blue. *O*-linked oligosaccharides were released by reductive β-elimination and analysed using chromatography/electrospray ion trap mass spectrometry.
Results:MG2 appeared to carry a consistent glycosylation between individuals, with a complex sialylation pattern

dominated by branched Si-Le^x epitopes. In contrast, MG1 glycosylation varied extensively between individuals, with regard to both terminal epitopes and the relative distribution of neutral and charged glycoforms. ABO-histoblood sequences were found on 'secretor' individuals both on neutral and sulphated oligosaccharides while 'non-secretors' did not have these epitope sequences and were characterised by a high degree of sialylation. Western-blot assays with antibodies confirmed this as an increased expression of Si-Le^a.
Conclusions: Our results highlight that the glycosylation between two salivary mucin glycoproteins are different. The hypervariation of the glycosylation of MG1 indicates that there is a component of the mucosal defence against pathogens that needs to be considered on an individual basis, while the more preserved glycosylation of MG2 indicates that there may be also a different, but more generic, use of mucins in pathogen protection.

P298
HISTOCHEMICAL DETECTION OF GLYCOLIPIDS IN NORMAL AND DIABETIC RAT KIDNEYS

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Histochemical localizations of various kinds of glycolipids were investigated with the use of confocal laser scanning microscopy and transmission electron microscopy. Normal Wistar rats and diabetic Goto-Kakizaki (GK) rats were prefixed by perfusion with 4% formaldehyde and 2.5% glutaraldehyde. Kidneys were isolated from the animals and cut into nonfrozen slices (50 micronmeter-thick). Samples were treated with monoclonal antibodies against globoside (Gb3) or gangliosides (GM1, GD3, o-acetyl-GD3 and GD1a) followed by treatments with Cy3-labeled secondary antibody for confocal laser microscopy and HRP-labeled secondary antibody for electron microscopy, respectively. In normal kidney cortex, each antibody revealed specific distribution pattern especially for glomerular basement membranes and for glomerular and urinary tubular epithelial cells. Among the antibodies, anti-Gb3 demonstrated increased stainability for the plasma membranes of podocyte foot processes in GK rats compared with those in normal Wistar rats. Specific distributions of glicolipids in kidney cortex seem to have some specific relationships with glomerular functions and may provide some important clues for understanding diabetic disorders.

P299
BILE-SALT-STIMULATED LIPASE AND MUCINS FROM MILK OF "SECRETOR" MOTHERS INHIBIT THE BINDING OF NORWALK VIRUS CAPSIDS TO THEIR CARBOHYDRATE LIGAND.

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Breast-feeding protection against calicivirus diarrhea is associated with the presence of high levels of 2-linked oligosaccharides in mother's milk and human calicivirus strains including the Norwalk virus (NV) use gut 2-linked fucosylated glycans as receptors suggesting the presence of decoy receptors in milk. Our aim was to analyse the ability of human milk to inhibit the attachment of recombinant Norwalk virus-like particles (rNV VLPs) to their carbohydrate ligand and to characterise potential milk inhibitors. Attachment of rNV VLPs to synthetic or natural carbohydrate ligands was inhibited by milk from mothers of defined secretor character. The inhibitory capacity of various milk fractions was assayed. Milk from women of the secretor phenotype was strongly inhibitory unlike milk from nonsecretor women which is devoid of 2-linked fucosylated structures. At least two fractions in human milk acted as inhibitor for the Norwalk virus capsid attachment. The first fraction corresponds to the bile salt-stimulated lipase and the second associates mucins MUC-1 and MUC-4. These proteins present tandemly repeated O-glycosylated sequences that represent decoy receptors for the Norwalk virus depending on the combined mother/child secretor status.

P300
GLYCOMIC ANALYSIS OF SNAKE VENOMS : IDENTIFICATION OF TERMINAL O-ACETYLATED DISIALYL MOTIF ON MULTIANTENNARY COMPLEX TYPE N-GLYCANS

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While the structures and functions of hundreds of snake venom proteins have been solved, similar investigation into their glycosylation has seldom been undertaken although its biological implications on the solubility, stability, and spreading rate of venom during envenoming is generally well appreciated. The venom glands are very specialized tissues characterized by fast evolution and great biodiversities. Based on a concerted strategy of MALDI-mass spectrometry (MS) profiling and MS/MS sequencing of permethyl derivatives, we have initiated a systematic glycomic analysis of the crude venoms from several closely related viperid species, particularly those from the genus *Protobothrops*, *Gloyidus*,

and *Deinagkistrodon*. In general, the N-glycosylation pattern of a genus is found to be typified by the non-reducing terminal epitopes carried on multiantennary complex type N-glycans. Focusing on the venom glycoproteins of *D. acutus* which are dominated by a characteristic terminal disialyl motif, subsequent studies demonstrated that the implicated N-glycans are mostly located on a serine protease, acutobin, Each of the antenna on a hybrid type and the bi-, tri- and tetra-antennary complex type glycans are substituted with a NeuAc2-8NeuAc2-3Gal- motif, making them highly sialylated. A second tier nanoLC-ESI-MS/MS analysis of glycopeptides further revealed that the disialyl motifs are O-acetylated and successfully defined the site specific glycosylation pattern, as well as the 2-8 linkage in conjunction with mild peridoate oxidation. This epitope is not only genus-specific but also tissue specific as indicated by its absence from
the serum glycoproteins.

P301
ROLE OF 9-O-ACETYLATED SIALIC ACIDS IN INDIAN VISCERAL LEISHMANIASIS

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Introduction: Although the presence of 9-*O*acetylated sialoglycoconjugates (9-*O*AcSGs) on the surface of erythrocytes and peripheral blood mononuclear cells (PBMC) of patients with VL ($PBMC_{VL}$) as novel biomarkers has been established, however, their role in the pathophysiology of the disease so far remained unexplored.
Materials and methods: The degree of complement-mediated hemolysis was compared with expression of 9-*O*AcSGs on erythrocytes. With an attempt to unravel the cellular and humoral responses of 9-*O*AcSGs, $PBMC_{VL}$ were stimulated with Achatinin-H, a 9-*O*AcSG binding probe. Both intracellular and secreted cytokines were measured. Immunogenecity of these newly induced 9-*O*AcSGs and their role in conferring host protection has been explored.
Results: Enhanced expression of linkage specific 9-*O*AcSGs on erythrocytes and $PBMC_{VL}$ using FITC-Achatinin-H was drastically reduced following effective anti-leishmanial therapy high lights their diagnostic and prognostic relevance. An excellent correlation was observed between the presence of 9-*O*AcSGs and the degree of alternate complement-mediated hemolysis. A significant amount of cellular response as reflected by enhanced mixed T_H1 and T_H2 cytokine expression was observed on stimulation of $PBMC_{VL}$ with Achatinin-H. We have observed an interesting correlation between 9-*O*AcSGs triggered cytokine response and anti-*O*AcSG antibody subclass expression. This 9-*O*AcSGs are highly immunogenic and capable of eliciting deposition of C3 as early as 3 minutes that triggered parasite lysis.
Conclusions: 9-*O*AcSGs on $erythrocytes_{VL}$ are potent complement-activators causing enhanced hemolysis via activation of the alternate pathway of complement, and may account for the significant degree of anemia, a common manifestation in VL. Among all the mitogens, only Achatinin-H was capable of triggering an enhanced lymphoproliferation, signifying, the unique potency of Achatinin-H in mediating mitogenic response of otherwise unresponsive $PBMC_{VL}$ suggesting an important immunomodulatory role of 9-*O*AcSGs which might possess the ability in mediating antibody class switching. Anti-*O*-AcSGs were identified as an important source of classical complement-activator even under normal physiological conditions suggesting their role in conferring host protection against parasite infection.

P302
EXPRESSION PATTERN OF HUMAN ST6GalNAc-I EVALUATED BY IMMUNOHISTOLOGY WITH NOVEL MONOCLONAL ANTIBODIES

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The mucin-type carbohydrate antigen, sialyl-Tn (Neu5Acα2-6GalNAc-O-Ser/Thr), is over expressed in many human cancers and associated with poor prognosis. The sialyl-Tn structure is synthesized by one of several α2,6sialyltransferases, which catalyze transfer of a sialic acid residue in α2,6-linkage to the GalNAcα1-O-Ser/Thr structure. Two sialyltransferases ST6GalNAc-I and ST6GalNAc-II have been identified as candidates for the synthesis of sialyl-Tn based on in vitro enzyme analysis, however, ST6GalNAc-I appears to be the most likely enzyme involved because its expression by in situ hybridization correlates with sialyl-Tn expression in human colonic cancer cells and transfection of ST6GalNAc-I but not ST6GalNAc-II induced sialyl-Tn expression in cell lines (Marcos et al, 2004). To gain further insight into the regulation of sialyl-Tn we have produced and characterized 2 mouse monoclonal antibodies to human ST6GalNAc-I. Recombinant soluble enzyme was expressed as secreted protein in High Five cells using the Bacculo-virus expression system and purified to near homogeneity with specific activity of 100 mU/ml and used as immunogen. Mabs 2C3 (IgG2a) and 1C9 (IgG1) were selected by immunoreactivity with transfected T47D and Sf9 cells expressing full coding or a secreted construct of human ST6GalNAc-I, and found to react in immunocytology/histology or by Western blot. ST6GalNAc-II and ST3Gal-I were negative controls. Application of these

antibodies revealed that the expression pattern of ST6GalNAc-I correlated well also at the protein level with that of sialyl-Tn in various human cancer cell lines and tissues (salivary glands, stomach and colon). The produced antibodies should be valuable for further studies of the biosynthetic basis for sialyl-Tn expression in cancer.

P303

β-HEXOSAMINIDASE AND ANTIGEN PRESENTING CELLS FUNCTION: IMPLICATION FOR NEURODEGENERATIVE PROCESS IN GM2 GANGLIOSIDOSIS

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Tay Sachs and Sandhoff diseases (GM2 Gangliosidosis), are a group of genetic progressive neurodegenerative disorders caused by a defective HEXA and HEXB gene respectively. The HEXA gene encodes for the alfa-subunit of the lysosomal enzyme beta-hexosaminidase (Hex) whereas the HEXB encodes for the Hex beta-subunit. The association of both subunits leads to the formation of the Hex A and Hex B isoenzymes. Absence either of Hex A or Hex B activity results in ganglioside GM2 and the neutral glycosphingolipid accumulation culminating with neurodeterioration from ages as early as 3–5 months. Unfortunately, the molecular pathways that underlie the neurodegenerative process are not yet fully understood. Recent studies, using either the animal model of these disorders or the post mortem brain of patients have shown a sustained inflammation process in the CNS, however whether neurodegeneration is the cause or consequence of inflammation must be yet defined.
We hypothesized that the genetic defects in the HEX genes may cause an altered immuno-system response which results in a sustained inflammation within the CNS of the animal model of GM2 gangliosidosis. Therefore, we investigated the Hex role in the immuno-system cells generating an *in vitro* model of human HEX genes null-CD1a-dendritic cells obtained in culture by hematopoietic stem cells differentiation. Our data clearly demonstrated a total inhibition of the Hex activity and a corresponding dramatic loss of function of these cells towards the activation of T lymphocyte proliferation response.
This work is supported by grants from Ministero Pubblica Istruzione (Italy): FIRB#; RBAU01M5FR_001; CIB-2004-2006.

P304

IMMUNOSUPRESSIVE EFFECT OF MUCINS ON B CELL FUNCTION IN TOMOR-BEARING STATE

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Many studies have suggested potential functions for Siglec 2 engagement of endogeneous ligands in regulating B cell function. In tumor-bearing state, epithelial cancer cells produce mucins and secrete them into cancer tissues and bloodstream of cancer patients, in which mucins may interact with Siglec 2. Because mucins have a variety of O-glycans with terminal α 2-6 linked sialic acid.
We isolated mucins from human colorectal cancer cells, LS 180 and mouse mammary adenocarcinoma cells, TA3-Ha. These mucins could bind to human and mouse recombinant soluble Siglec 2, respectively. LS 180 mucin bound to Burkit lymphoma, Daudi cells and to Cos 7 cells expressing wild type Siglec 2 but not ectodomain-mutated or –deleted Siglec 2. Next, we investigated the effect of mucins on BCR-mediated signal transduction by using Daudi cells and Siglec 2 cDNA transfected mouse B cell. These cells were stimulated with anti IgM F(ab')2 in the presence of mucins and cell lysate was prepared. Siglec 2 immunoprecipitates were subjected to SDS-PAGE followed by western blotting. Ligation of mucins to Siglec 2 reduced the tyrosylphosphorylation and recruitment of SHP-1. It is noted that mucin engagement also diminished the phosphorylation of MAPK in a dose dependent manner. These results are different from the fact that BCR mediated signal transduction is potentiated by anti Siglec 2 Ab engagement due to sequestering of the phosphatase. One possibility is that mucins bridge so many receptors on the cell surface that the lateral movement of the receptors including BCR may be affected.

P305

EFFECT OF MUCIN ON FUNCTIONAL ASPECT OF DENDRITIC CELL

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Dendritic cells (DC), which are the most potent professional APCs of the immune system, play an important role in the initiation of adaptive immunity. Siglecs are sialic acid –binding Ig-like lectins and most siglecs possess ITIM in the cytoplasmic region. Their endogenous ligands, however, have not been elucidated. Epithelial tumor cells produce mucins carrying a large number of O-glycans with terminal sialic acids and secrete them into bloodstream. Thus, it is

conceivable that mucins are preferential ligands for siglecs in tumor-bearing state. We are interested in the effect of mucins on DC function.
Peripheral blood monocytes were isolated from PBMCs of healthy donors by using $CD14^+$ magnetic beads and differentiated to mature DC as described previously (J Immunol 170; 4069, 2003). During the development of monocytes to DC, expression of Siglec 3 and 7 was diminished or unchanged, whereas expression of Siglec 9 was elevated. Recombinant soluble Siglec 3 could bind to mucins isolated from human colorectal cancer cells, LS 180 (LSM) preferentially and to bovine submaxillary mucin (BSM) and poly α2,6/2,3 sialyl LacNAc weekly. Recombinant soluble Siglec 9 could bind to LSM and poly α2,3 sialyl LacNAc preferentially and to BSM moderately. It is noted that during the development, DC was diminished by the treatment with LSM and annexin V-binding cells were increased. In addition, treatment with LSM decreased the LPS-induced IL-12 production from DC.
These results suggest that mucins down-regulate immune responses.

P306
THE ROLES OF LACCER-ENRICHED GLYCOSIGNALING DOMAIN IN NEUTROPHIL FUNCTIONS(II) -ROLE OF FATTY ACID CHAINS OF LACCER ON PHAGOCYTOSIS-

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Human neutrophils play the first line of defense against invading microorganisms and an important role in acute inflammatory reaction. They exert their bactericidal activities through the mechanisms that lead to the destruction of microorganisms. In mature neutrophils, lactosylceramide (LacCer, CDw17) forms microdomains (LacCer-enriched glycosignaling domain, GSD) coupled with a Src family kinase Lyn on plasma membrane. Previously, we demonstrated that the ligand binding to LacCer activated neutrophils through Lyn activation in LacCer-enriched GSD. LacCer has hydrophilic sugar moiety and hydrophobic ceramide. The ceramide is composed of sphingosine and several lengths of fatty acid chains. However, LacCer is not located in the cytoplasmic leaflet of membrane bilayer. It remains obscure how LacCer activates Lyn. Interestingly, DMSO-differentiated HL-60 cells do not have LacCer-enriched GSD-mediated signal transduction cascades. We show here that long fatty acid chains-containing LacCer significantly enhanced the phagocytosis of not only non-opsonized zymosan but *Mycobacterium avium complex* (*MAC*) by DMSO-treated HL-60 cells. In contrast, short fatty acid chains-containing LacCer did not affect phagocytosis of those cells. Moreover, the phagocytic enhancement was completely inhibited by Src family kinase inhibitor PP1 and PI_3 kinase inhibitors LY294002 and wortmannin. On the other hand, negative control compounds PP3 nor LY303511 did not enhance the HL-60 cell phgocytosis of those microorganisms. Those data suggest that the phagocytosis of non-opsonized microorganisms is mediated by LacCer-enriched GSD and long fatty acid chain of LacCer was involved in neutrophil phagocytosis.

P307
LEWIS X-OLIGOSACCHARIDES APPEARED AT SEGMENTATION PERIOD OF ZEBRAFISH EMBRYO

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The Lewis x epitope is known as stage specific embryonic antigen-1. We previously reported the isolation of the gene of zebrafish α(1,3)fucosyltransferase (zFT1) capable to synthesize the Lewis x structure[1)]. The gene was expressed specifically in segmentation period. Meanwhile, we also reported the complex type *N*-glycans significantly appeared from the segmentation period of zebrafish embryo[2)].
In the present study, we have tried to detect the enzyme products of zFT1 in the embryo. Oligosaccharides were isolated from the zebrafish embryos followed by pyridylamination. The PA-oligosaccharides that were α1-3/4fucosidase sensitive and expressed time-dependently at 18 h after fertilization were picked up as candidates for the *in vivo* products synthesized by zFT1. The structures of these oligosaccharides were determined to be a biantennary complex-type with a couple of Lewis x structures. Remarkably, majority of these oligosaccharides was detected as free form. Furthermore, the endo-β-*N*-acetylglucosaminidase activity was detected in the 18 h embryo. These results suggest that the appearance of oligosaccharides synthesized by zFT1 occurred in the embryo at segmentation period as a free form due to liberation from glycoproteins by digestion with endo-β-*N*-acetylglucosaminidase and/or glycoamidase.
1) Kageyama, N., Natsuka, S., & Hase, S. (1999) J. Biochem., 125, 838-845.
2) Takemoto, T., Natsuka, S., Nakakita, S., & Hase, S. (2005) Glycoconj. J., 22, 21-26.

P308
ROLES OF MAJOR CARBOHYDRATES ON CRY J 1, THE MAJOR ALLERGEN OF CRYPTOMERIA JAPONICA POLLEN, IN HUMAN IGE AND T CELL RESPONSES.

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Introduction: We have demonstrated that carbohydrates in Cry j 1, the major allergen of *Cryptomeria japonica* pollen, play a major role in promoting Cry j 1-specific Th2 response. However, little is known as to whether the carbohydrates directly participate in allergic responses. We sought to determine whether Cry j 1-related oligosaccharides function as IgE and/or T cell epitopes. In addition, the regulatory effect of Cry j 1-related oligosaccharide on Cry j 1-specific T cell responses was investigated.
Experimental approaches: Two monovalent oligosaccharides largely found on Cry j 1, Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc (M3FX), and GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc (GN2M3FX) were prepared. Manα1-2Manα1-6(Manα1-2Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc (M9A) was used as control. Competitive inhibition ELISA for Cry j 1-specific IgE was performed using these oligosaccharides as inhibitors. In addition, T cell lines specific for Cry j 1 were established, and cellular responses against these oligosaccharides were investigated in the presence or absence of the respective antigens.
Results: Overall, neither M3FX or GN2M3FX displayed inhibitory effect on the binding between IgE and Cry j 1. In addition, M3FX did not by itself stimulate Cry j 1-specific T cells. However, M3FX significantly inhibited Cry j 1-induced proliferation and IL-4 production in Cry j 1-specific T cells.
Conclusion: These results suggest that Cry j 1-related oligosaccharides are not major epitopes for as IgE or T cells, however, these oligosaccharides have a novel potential to inhibit Cry j 1-specific T cell responses selectively.

P309
EXPLORING INTESTINAL IMMUNE SYSTEM AND MAST CELL REGULATION

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introduction: Small intestinal mucins in rats infected with nematode *N.brasiliensis* undergo transient changes among their terminal *O*-glycan epitopes. We hypothesise that this is a general response to intestinal infections. However, the initial sensors of infectious agents in the intestine would be inflammatory cells secreting mediators upon activation, rather than the mucin producing goblet cells. In *N.brasiliensis* infection, one of those is the mast cell. To learn more about mast cell regulation, we investigated intracellular *O*-GlcNAc and *O*-phosphate protein modifications in a mast cell model system.
experimental: Mice were infected with *N.brasiliensis* or *C.jejuni* to investigate if glycosylation alterations generally occur upon infection. Mucin *O*-glycans were isolated from small intestines of infected and control mice, and analyzed using MS. As a mast cell model, RBL-2H3 cells were activated via cell surface IgE receptors. Cell lysates were prepared and subjected to western-blotting using antibodies against *O*-phosphate and *O*-GlcNAc epitopes.
results: Infecting mice with pathogens induced production of blood group H epitopes, due to upregulation of the Fut2 glycosyltransferase. Activation of RBL-2H3 cells induced changes in *O*-phosphate but not *O*-GlcNAc modifications on intracellular proteins.
conclusions: The hypothesis of a general response to infections was supported, with glyco-profiling showing alterations in mucin *O*-glycosylation in different model systems. The investigation of mast cell regulation indicated that protein phosphorylation has an important function, as expected. However, no changes in *O*-GlcNAc modification of proteins were observed. A counteracting relation between *O*-phosphate and intracellular *O*-GlcNAc has been suggested, but this hypothesis was not supported in our model system.

P310
***Mannheimia haemolytica* ADHESIN ACTIVATES BOVINE NEUTROPHILS OXIDATIVE BURST THROUGH GlcNac SPECIFIC RECEPTORS**

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In this work we assessed the specificity and biological effect of the Mannheimia haemolytica purified adhesin (MhA) on bovine leukocytes. Histochemical staining and flow cytometry showed that MhA recognizes specifically monocytes (CD14+) and neutrophils (CD16+), but not Iymphoid cells. MhA induced oxidative response in purified neutrophils; this eftect was 1.3-1.5-fold higher than the effect observed or with lectin WGA (wheat germ agglutinin) which shared similar sugar specificity than MhA. The induced reactions and cellular recognition by MhA were inhibited with GlcNAc and its oligomers; as well as by glycoproteins containing tri-and tetra-antennary N-glycosydically linked glycans. Our findings suggest that neutrophils are the main target for MhA, and induce production of oxidative radicals, which seem to play an important role in tissue damage during mannheimiosis.

P311
SPECIFIC INTERACTIONS OF HUMAN GALECTINS WITH TRYPANOSOMA CRUZI.
II.- EXPRESSION CHANGES DURING MURINE INFECTION.

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Trypanosoma cruzi, an obligate intracellular protozoan parasite, with a complex life cycle, it is able to infect a large variety of nucleated cells and is the causative agent of Chagas´ disease, pathology that affects more than 17 million people mainly in South America, and characterized by inflammation and degeneration of cardiac and other tissues. It has been shown that host resistance to experimental Chagas´ disease depends on both innate and acquired immunity and that immune response has been associated with both protection and pathogenesis. Mice infected with T. cruzi resemble human disease as develop detectable parasitemia during acute infection, which is the followed by chronic tissue parasitism. However, there is known that different strains of inbred mice show different patterns of susceptibility and resistance to the infection. The basis of resistance is not known, but they exhibit different patterns of cytokine response to the infection, suggesting differential modulation of Th1/Th2 dichotomy. Recent evidence implicates Galectins as regulators of immune homeostasis by acting at innate and adaptative responses and by influencing the Th1/Th2 cytokine balance. We report here the results of studying the expression level of galectins 1, 3 and 9 in the spleen, heart, thymus, and peripheral ganglia of mice BALB/c and C57BL/6 (naturally susceptible and resistant to infection) during the course of experimental infection by T. cruzi. The results indicate different basal expression levels of galectin 1 and 3, and opposite direction in the changes of expression during the course of the infection in immune tissues. Regarding galectin 1 in the heart, it is shown a peak of expression at the end of acute phase in the resistant mice compared with a constant increase in the susceptible mice. The results will be discussed considered an extension of Th1/Th2 dichotomy to the galectin expression levels.
The financial support of Spanish Science and Industry Ministry through Research Project SAF2002-02444 is acknowledged.

P312
DETECTION OF TRANSFERRIN SIALOFORMS IN CEREBROSPINAL FLUID (CSF) BY CAPILLARY ELECTROPHORESIS (CE).

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Asialotransferrin (0-Tf) is an accepted marker of CSF leakage from the subarachnoid space into the nasal or aural cavity as the result of a head trauma. Nowadays, the 0-Tf detection method of choice is isoelectric focusing on polyacrylamide gel with direct immunofixation of transferrin and silverstaining. A great disadvantage is the time-consuming technique (> 5 h.) while the clinician needs a quick result in order to treat the possibility of central nervous system infection. The aim of this study was to evaluate the use of CE in the detection of 0-Tf in CSF. CE has already been used for the detection of transferrin sialoforms in blood of patients suspected of chronic alcohol abuse. An increase in 0- and 2-sialotranferrin is suspicious for alcohol abuse. The concentration of transferrin in blood varies from 2.0 – 4.0 g/L which is a 100-fold higher than the transferrin concentration in CSF. To overcome the difference in concentration we applied a rapid (15-30 min) centrifugation concentration step that was followed by CE analysis (8 min) for the detection of transferrin sialoforms. Migration times of the different transferrin sialoforms of CSF were determined by CE analysis of transferrin after partial sialidase treatment and were comparable to those in serum. A detection limit of 4 g/L total protein after concentration was determined by CE analysis of transferrin in mixtures of CSF and distilled water. With this method it is possible to detect the 0-Tf in excretions from the nose or the ear within 45 min.

P313
MOUSE HOMOLOGS THAT SHARE LIGAND-BINDING AND ENDOCYTIC ACTIVITIES WITH HUMAN DC-SIGN

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The human receptors DC-SIGN and DC-SIGNR are of interest because of their potential roles in cell adhesion and pathogen recognition and because they can enhance the efficiency of human immunodeficiency virus infection by presenting the virus to T cells. Although closely related to each other in sequence, these two receptors differ in ligand-binding and intracellular trafficking activities. Screening of the mouse genome sequence reveals seven potential homologs of the human receptors, some of which have not been previously described. The biochemical and cell biological properties of these mouse SIGNs have been investigated in order to define functional orthologs of the two human receptors. The SIGNR6 gene is not expressed, while SIGNR4 appears to lack sugar-binding activity. Thus, only five of the mouse proteins are expressed and bind mannose-containing ligands. Like human DC-SIGN, three of these proteins show preferential binding of fucose over mannose and release ligand at endosomal pH. However, only two of them, SIGNR1 and SIGNR3, are able to mediate endocytosis and degradation of

neoglyoprotein ligands. Testing on a glycan array reveals that mouse SIGNR3 also closely resembles human DC-SIGN in ligand-binding profile. Thus, mouse SIGNR3 is the mouse protein most like human DC-SIGN. Mouse SIGNR0, previously designated 'mouse DC-SIGN', actually resembles human DC-SIGNR as it binds preferentially to mannose and does not display endocytic activity. SIGNR2, the final homolog, is a soluble protein that binds preferentially to N-acetylglucosamine-containing ligands and may have a role in innate immunity analogous to serum mannose-binding protein.

P314
PARTIAL ACTIVATION OF HUMAN PLATELETS BY GALECTIN-3

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Activation of thrombocytes is an essential step in haemostasis. Activated platelets release microvesicles. Galectin-3 is a β-galactoside-binding protein implicated in diverse biological processes. In this study, we observed that human galectin-3 added extracellular to washed human platelet induced microvesicles formation, which was demonstrated by protein S and Annexin V binding assay. Glycoprotein IIb-IIIa (integrin$\alpha_{IIb}\beta_3$) is the most abundant glycoprotein on platelet plasma-membranes and binds to adhesive proteins via the recognition of short amino acid sequences—RGD motif. Platelet vesiculation requires intact glycoprotien IIb-IIIa since de-complexation of surface glycoprotien IIb-IIIa receptors led to an inability of platelets to vesiculate in response to stimulation with collagen. By combined affinity purification, mass fingerprinting and immunoblot glycoprotein IIb-IIIa was identified as one of galectin-3 ligands in platelets. Galectin-3 treatment induced slight conformational changes in glycoprotein IIb-IIIa, indicated by PAC1 binding increased from 10.78% to 19.08%. Galectin-3 did not influence platelet aggregation nor inhibit the aggregation induced by ADP, but galectin-3 induced agglutination of washed platelets. Adhesion of platelets can be observed on a galectin-3 coated plate under flow through system. Effects of galectin-3 on platelets could be inhibited by lactose in a dose dependent way.

P315
SYNTHETIC AND EVOLUTIONARY APPROACHES TOWARDS A CARBOHYDRATE IMMUNOGEN FOR HIV-1

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A small number of antibodies, isolated from HIV-infected individuals, provide broad sterilising immunity against viral challenge in animal models. We have previously characterised the epitope of 2G12, a neutralizing anti-HIV IgG1 that binds to a cluster of Manα1-2Man residues within the N-linked glycans of HIV-1 gp120. An immunogen, capable of eliciting 2G12-like antibodies, would therefore confer protection against natural HIV infection. However, the inherently poor immunogenicity of the 'self' oligomannose glycans of gp120 is a major barrier in the design of carbohydrate vaccine for HIV-1.
Using sera from healthy and infected patients, we show that whilst the oligomannose glycans ($Man_{5-9}GlcNAc_2$) of gp120 are not antigenic, synthetic mannosides, sharing extensive structural identity with 'self' sugars, are recognized by the humoral immune system. Furthermore, these synthetic antigens bind to 2G12 as efficiently as the natural oligomannose ligand (Ki = 0.5mM). Multivalent conjugates show a further enhancement in binding to 2G12 (Ki = 0.5uM). We are also pursuing an evolutionary approach towards an HIV immunogen. Yeast mannans are highly immunogenic but are not significantly antigenically cross-reactive with mannoses found on HIV-1. However we have demonstrated that 2G12 can bind to mannan from *S.cerevisiae* deficient in α1-3 mannosyl transferase (Mnn1p). The exposure of clustered Manα1-2Man residues on the ΔMnn1 strain is consistent with the known structure of the 2G12 epitope. We are currently subjecting *S.cerevisiae* ΔMnn1 to repeated rounds of mutation and selection for 2G12 binding. Preliminary results indicate that binding to 2G12 (and hence potential antigenic mimicry of HIV) is a selectable phenotype.

P316
ISOLATION OF THE RECEPTOR FOR WHEAT GERM AGGLUTININ FROM HUMAN NEUTROPHILS

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Neutrophils participate in host protection and central to this process is the regulation of oxidative mechanisms. We purified by affinity chromatography the receptor for the GlcNAc-specific WGA from CD14+CD16+ cell Iysates. The WGAr is a glycoprotein composed by two subunits of 78 and 63 kDa. It is a glycoprotein composed mainly by Ser, Asx and Gly; in minor proportion His, Cys and Pro; in its glycannic portion it contained GlcNAc, Gal, Man, NeuAc and GaINAc.

The amino acid sequence was determined from tryptic peptides by MALDI-TOF, both subunits showed homology with cytoqueratin type II (26%) and human transferrin receptor (16%). Antibodies against WGAr induces higher oxidative burst than WGA, determined by NBT reduction; however, this effect was inhibited with 200 mM GlcNAc and 200 mM glycine ($p<0.01$) than dipyrone, suggesting WGAr participates as mediator in signal transduction for Ca++ mobilization or permeabilization in neutrophils.
Financed in part by PAPIIT-UNAM.

P317
MANNOSE BINDING LECTIN (MBL) BROADLY NEUTRALIZES HIV-1 CLADE B, B', E, D

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Introduction
Mannan binding lectin (MBL) is one of the important factors in humoral immune system. MBL has been reported to have the biological activity against various microbes such as influenza A virus. In this study, neutralization activities of MBL against a panel of human immunodeficiency virus (HIV) have been investigated.
Experimental approaches
HIV-1 strains Th14, JRCSF, LP65, NDK were used for the assay. Inhibitory activity of MBL against HIV was investigated by using PBMC and GHOST cell in the presence of various concentrations of native and recombinant human MBL. In the PBMC assay, 100 TCID50 HIV-1 were used for the infection, followed by the quantification of P24 after seven days incubation. In GHOST cell assay, reduction of GFP positive cell number was measured after four days incubation.
Results
Both the native and the recombinant MBL could neutralize HIV-1 strains Th14, JRCSF, LP65, NDK. The concentration(s) enabling 50 % inhibition of HIV infectivity in both native and recombinant MBL were less than 10 micro-g/ml. In addition, anti-MBL monoclonal antibody can inhibit the virus-neutralization effect of MBL against HIV-1 in PBMC-based assay.
Conclusions
MBL exercised the ability to inhibit the infectivity of HIV-1 in the direct neutralization test. This phenomenon was completely different from the neutralization by the anti-HIV-1-monoclonal antibodies specific for clade or subclade. MBL might become one of the therapeutic agents that can get over the wall of clade of HIV-1.

P318
FINE ESPECIFICITY OF MONOCLONAL ANTIBODY MEST-3: POSSIBLE USE OF ANTI-GIPC MONOCLONAL ANTIBODIES IN FUNGAL IMMUNOTHERAPY

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The general increase in the risk of fungal infection, particularly in immunocompromised patients from organ transplants, HIV infections, and cancer has raised the interest in identifying new targets for fungal immunodiagnosis and therapy. Recently, data showing that fungi are vulnerable to glycosphingolipid (GSLs) biosynthesis inhibitors set grounds in fungal GSLs as potential targets for antifungal agents. Particularly interesting targets are the fungal glycoinositol phosphorylceramides (GIPCs) which are highly susceptible to inositol phosphorylceramide synthase inhibitors which are remarkably toxic to many mycopathogens, but exhibit low toxicity in mammals.
An IgG2a mAb directed to *Paracoccidioides brasiliensis* GIPC Pb-2 (Man*p*α1→3Man*p*α1→2Ins1-P-1Cer) was established and termed MEST-3. HPTLC immunostaining, and solid-phase radioimmunoassay showed that MEST-3 reacts with GIPCs from yeast forms of *P. brasiliensis*, *Histoplasma capsulatum*, and *Sporothrix schenckii* and hyphae of *Aspergillus fumigatus*. The specificity of mAb MEST-3 was assessed using methyl-monosaccharides, Man*p*α1-2Man*p*, Man*p*α1-3Man*p*, Man*p*α1-6Man*p*, Man*p*α1-3Man*p*α1-2Ins (Pb-2 oligosaccharide) and Man*p*α1→3Man*p*α1→6Ins1 (Ss-M2 GIPC oligosaccharide from *S. schenckii*) as inhibitors of MEST-3 binding to Pb-2. No inhibition was observed with methyl-monosaccharides or with Ss-M2 oligosaccharide. Only Man*p*α1-3Man*p* and Pb-2 oligosaccharide at 100mM were able to inhibit about 95% MEST-3 binding to Pb2 antigen, indicating that the epitope recognized by MEST-3 is Man*p*α1-3Man*p*α1-2.
Preliminary studies detected an inhibitory activity of anti-GIPC mAbs on fungal growth and/or colony formation, these data clearly point out the importance of these mAbs as new tools in antifungal immunotherapy.
Supported by: CAPES, CNPq and FAPESP

P319
LIPOPOLYSACCHARIDE OF *COXIELLA BURNETII*. STRUCTURAL MOTIFS INVOLVED IN THE IMMUNOBIOLOGY OF Q FEVER

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Coxiella burnetii is the etiological agent of Q fever, a zoonosis that is endemic throughout the world. The disease can occur as an acute disabling illness or develop towards a chronic form. Lipopolysaccharide (LPS) is a major determinant of virulence expression and infection of the bacterium. Its composition and structure determine to a great extent interactions between the microorganism and host including pathogenicity and immunogenicty of the agent. Thus, the structure/function relationship studies are of potential interest.
From virulent *C. burnetii* phase I cells an LPS I was isolated by a hot phenol/water method. It was lipid A deprived and separated by HPLC. The isolated polysaccharide fractions were analyzed for their chemical composition and structure by methylation analysis in combination with GC-, MALDI-ToF- and ESI-MS techniques. The selected, structurally characterized polysaccharide antigens were assayed in ELISA with anti-*C. burnetii* phase I and II rabbit sera and with patient's sera.
We have found that LPS I contains chemically distinct populations of the O-polysaccharide chains differing in their antigenic reactivities. The chains contain a variable amount of two unusual sugars virenose (Vir) and dihydrohydroxystreptose (Strep) that are unevenly distributed along them in terminal positions. The polymer backbone is predominatly composed of GlcNAc residues. A remarkable decrease in the serological activity of O-specific chains with anti-*C. burnetii* phase I and II sera was observed when Vir and Strep were selectively removed from them. These and other data indicate that both sugars are involved in the immunobiology of Q fever.

P320
COMMON POLYMORPHISMS IN HUMAN LANGERIN AFFECT STABILITY AND SUGAR-BINDING ACTIVITY

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Langerhans cells are specialized skin dendritic cells that take up and degrade antigens for presentation to the immune system. Langerin, a cell surface C-type lectin of Langerhans cells, can be internalized and accumulates in Birbeck granules, sub-domains of the endosomal recycling compartment that are specific to Langerhans cells. Langerin binds glycoconjugates containing mannose and related sugars and is able to mediate uptake and degradation of such ligands. Analysis of the human genome has identified three single nucleotide polymorphisms as well as one rare mutation that result in amino acid changes in the carbohydrate-recognition domain of langerin. These changes have the potential to alter the activity of langerin and thus might affect susceptibility to infectious diseases caused by micro-organisms that contain surface glycans that interact with langerin. The effects of the amino acid changes on the activity of langerin were examined by expressing each of the polymorphic forms. Expression of full-length, membrane-bound, versions of the four common langerin haplotypes in fibroblasts revealed that all of these forms can mediate uptake of neoglycoprotein ligands. However, sugar binding assays and differential scanning calorimetry performed on fragments from the extracellular domain showed that some of the amino acid changes have large effects on the affinity of the carbohydrate-recognition domain for mannose and on the stability of the extracellular domain. These findings suggest that certain langerin haplotypes may differ in their binding to pathogens and thus be associated with susceptibility to infection.

P321
CHANGES IN GLYCOSYLATION INDUCED BY MUTATION OF POTENTIAL N-GLYCOSYLATION SITES OF DUFFY ANTIGEN/RECEPTOR FOR CHEMOKINE

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The Duffy antigen/receptor for chemokine (DARC) is a seven-transmembrane glycoprotein carrying the Duffy (Fy) blood group and erythrocyte receptor for the malarial parasite *Plasmodium vivax.* In addition to red blood cells, DARC is expressed in post-capillary venules of endothelial cells in most tissues and in some epithelial cells. The polypeptide chain of DARC contains two NSS motifs at positions 16 and 27 and one NDS motif at position 33 that represent canonical sequences for efficient N-glycosylation to asparagine residues. The present study addressed the following question: are all three canonical N-glycosylation sites on the Duffy protein used? To answer this question we generated N-glycosylation variants in which potential N-glycosylation sites (AsnXSer) were removed by replacement of serine by alanine. We obtained seven glycosylation mutants of DARC: three single mutants (S18A, S29A, S35A), three double mutants (S18A.S29A, S18A.S35A, S29A.S35A), and a triple mutant (S18A.S29A.S35A). cDNA encoding DARC mutants were cloned into the eukaryotic expression vector pcDNA3.1myc/HisA and expressed in human K562 cells. Recombinant Duffy protein level on stable transfectants was monitored by flow cytometric analysis using anti-Fy6 antibody. K562 cell transfectants expressing wild-type or mutated forms of Duffy were then lysed with CelLytic buffer, purified by metal affinity chromatography on Ni-NTA agarose, and subjected to Western blott analysis with an anti-Duffy monoclonal antibody (anti-Fy6) and an anti-myc monoclonal antibody (9E10). Elimination of N-linked glycosylation sites produced lower-molecular-weight recombinant proteins. The gel electrophoreisis data indicate that all three canonical sites are used for sugar attachment.

P322

STRUCTURES, SIGNAL MECHANISM FOR IMMUNOLOGICAL PRO-IL-1 BIOACTIVITY, AND *E.COLI* LPS TOLERANCE OF PHOSPHOGLYCOLIPIDS FROM THE THERMOPHILE *MEIOTHERMUS TAIWANENSIS* ATCC BAA-400

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The structures of two major phosphoglycolipids from the thermophilic bacteria *Meiothermus taiwanensis* were determined using spectroscopic and chemical analyses to be 2′-*O*-(1,2-diacyl-*sn*-glycero-3-phospho)-3′-*O*-(α-*N*-acetyl-glucosaminyl)- *N*-glyceroyl alkylamine (PGL1) and the novel structure 2′-*O*-(2-acylalkyldio-1-*O*-phospho)-3′-*O*-(α-*N*-acetylglucosaminyl)-*N*-glyceroyl alkylamine (PGL2). PGL2 is the first phosphoglycolipid identified with a 2-acylalkyldio-1-*O*-phosphate moiety. The fatty acids of the phosphoglycolipids are mainly *iso*-$C_{15:0}$ and -$C_{17:0}$, and *anteiso*-$C_{15:0}$ and -$C_{17:0}$.
The phosphoglycolipids could selectively up-regulate prointerleukin-1β (proIL-1) expression of human monocyte THP-1 cells in a dose-dependent manner, primarily by the PTK/PKC/MEK/ERK pathway through the stimulation of Thr-202/Tyr-204-phosphorylated ERK1/2. However, the phosphoglycolipids activated p38 and c-Jun phosphorylation in a weak stimulation.
In medical application, according to the similarities between phosphoglycolipids and lipopolysaccharides, the approach was done for evaluation the lipopolysaccharides adaptation/tolerance which is defined as the prolonged exposure of human monocytes to bacterial LPS induce a state of adaptation/tolerance to subsequent LPS challenge. The report proteins pro-IL1, IL-1, and TNF-α were selectively repressed and in a dose-dependent pattern after phosphoglycolipids treatments. The IL-1R-associated kinase (IRAK) protein, especial in ERK1/2 phosphorylation was meanwhile very closely correlated with the development of *E.coli* LPS tolerance.

P323
THE ROLES OF LACCER-ENRICHED GLYCOSIGNALING DOMAIN IN NEUTROPHIL FUNCTIONS (I) -VISUALIZATION OF LACCER-MEDIATED NEUTROPHIL PHAGOCYTOSIS-

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Microdomains (lipid rafts) are defined biochemically as detergent-insoluble fractions. Therefore, if some detergent-soluble molecules are contained in the “lipid rafts”, they cannot be recovered biochemically in the “lipid raft” fractions from living cells. Therefore, we need new methods for elucidating real features of lipid rafts. Here we report visualization of lactosylceramide (LacCer) in living neutrophils and the molecular dynamics of LacCer during the neutrophil phagocytosis. LacCer is thought to cluster with sphingomyelin, cholesterol and transducer molecules on plasma membrane to form LacCer-enriched glycosignaling domain (GSD) which is a kind of lipid rafts. Recent studies indicate that LacCer in GSD works as an adhesion molecule and plays an important role in binding microorganisms and activating neutrophils. BODIPY-LacCer is a fluorescent analog of LacCer prepared from D-erythro-sphingosine and therefore has the same stereochemical conformation as biologically active, natural LacCer. Larger amount of BODIPY-LacCer was incorporated into LacCer-, GarCer-, GM1-, or GM3-containig liposomes than into phosphatidylcholine liposome. In contrast, BODIPY-LacCer was hardly incorporated into sphingomyelin-containing liposomes. Co-localization of the BODIPY-LacCer and antibody-detected LacCer was observed in the BODIPY-LacCer-treated neutrophils, suggesting that BODIPY-LacCer was incorporated into the authentic LacCer-enriched microdomains. Time-lapse confocal microscopy revealed that BODIPY-LacCers in the neutrophils incubated with zymosan made uniform clusters on the phagosome membranes and showed rapid movements, however, BODIPY-LacCers in the neutrophils incubated with *Mycobacterium avium complex (MAC)* made larger clusters around *MAC* and showed impaired movements. These results demonstrate the usefulness of BODIPY-LacCer for visualizing the molecular dynamics of the LacCer-enriched GSD during the neutrophil phagocytosis.

Patology of glycosylation and deglycosylation

P324
GLYCOFORM OF N-LINKED OLIGOSACCHARIDE OF TONSILLAR IGA1, AND ABERRANT IGA1 PREPARED FROM THE SERUM BY ASIALO-, AGALACTO-IGA1-SEPHAROSE COLUMN AND THEIR INVOLVEMENT IN IGA NEPHROPATHY

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Introduction: There are many reports on the presence of an incomplete O-linked oligosaccharide of IgA1 in some IgA nephropathy patients. As the candidates of such an IgA1, the tonsillar IgA1 and aberrant IgA1 in the IgA1-binding protein (IgA1-BP) separated from serum by asialo-, agalacto-IgA1-Sepharose were proposed. The aberrant IgA1 in IgA1-BP was thought to be the same molecule as the deposited IgA1 in an IgA nephropathy patient since a compositional similarity between IgA1-BP and glomerular depositied protein was reported.
Experimental procedure: Analysis of the N-glycan glycoforms of IgA1 was carried out using the three-dimensional mapping method.
Results: The sugar chain composition was almost the same for these IgA1 preparations. However, the structural characteristics for the aberrant IgA1 showed a drastic increase in the neutral N-glycans, especially, 25 % of the sugar chains of the aberrant IgA1 was the high mannose-type as compared to approximately 5-6 % in the serum IgA1 and tonsillar IgA1. The neutral complex-type N-glycan chain with fucose was higher in both the aberrant IgA1 and tonsillar IgA1 than in the serum IgA1.
Conclusion: We found abnormality in the N-linked oligosaccharides of the aberrant IgA1. These results indicated the possibility that the aberrant IgA1 could be derived from tonsillar tissue due to their incomplete O-glycan and N-glycan chains. Meanwhile, the abundance of the high-mannose type sugar chain in the aberrant IgA1 might induce the activation of the mannan-binding lectin (MBL)-mediated complement pathway in the mesangium region. There was evidence for the MBL-induced complement activation in the IgA nephropathy.

P325
ELEVATED POST-TRANSLATIONAL MODIFICATION OF PROTEINS BY O-LINKED N-ACETYLGLUCOSAMINE IN VARIOUS TISSUES OF DIABETIC GOTO-KAKIZAKI RATS ACCOMPANIED BY DIABETIC COMPLICATIONS

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The hexosamine biosynthetic pathway is one of the possible mechanisms involved in diabetic complications. We aimed to examine the changes of O-linked N-acetylglucosamine (O-GlcNAc) modification of proteins in diabetes, especially in the pathogenesis of diabetic complications. The expression of O-GlcNAc-modified proteins and O-GlcNAc transferase was examined immunohistochemically in various tissues (sciatic nerve, kidney, retina, liver, skeletal muscle, white adipose tissue, skin, cornea) of diabetic Goto-Kakizaki (GK) rats which has spontaneous development of non-insulin-dependent diabetes, and nondiabetic Wistar rats. In all of the tissues of the non-diabetic Wistar rats, the O-GlcNAc and O-GlcNAc transferase immunoreactivities were observed in almost all cells, with strong expression indicated in the nucleus and weak expression in the cytoplasm. In the diabetic GK rats, these immunoreactivities in the sciatic nerve, kidney, liver, and cornea increased in intensity. In these tissues, morphological abnormalities accompanied by neuropathy, nephropathy, hepatic steatosis and keratopathy were observed. In contrast, immunoreactivities in the other tissues did not change in the diabetic GK rats. Furthermore, no morphological changes were observed in these tissues. The treatment of Wistar rat corneas with O-(2-acetamide-2-deoxy-D-glucopyranosylidene) amino-N-phenyl-carbamate (PUGNAc), an inhibitor of O-GlcNAcase, the enzyme that removes O-GlcNAc from proteins, increased the level of O-GlcNAc and caused a decrease in the number of hemidesmosomes and the detachment of corneal epithelial cells from the basement membrane. These results indicate that the elevated expression of O-GlcNAc-modified proteins may play a causative role in the diabetic complications seen in diabetic GK rats.

P326
TARGETING IgG GALACTOSE LOSSES IN RHEUMATOID ARTHRITIS

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Decreased levels of serum IgG galactosylation are typically observed in rheumatoid arthritis [1-3]. The study of such alterations in pathological IgG is a powerful tool for achieving an early diagnosis of the mentioned disease.

In the present work, a simple and reliable method for detecting galactose losses from IgG is reported. It is based on the specific oxidation of IgG galactose residues (with galactose oxidase), and subsequent linkage of a fluorescent tag with the formed aldehyde group on C-6 galactose.
Among the investigated tagging strategies, linking the fluorophoric agent Alexa 488® to oxidized galactose has turned out to be the most sensitive and reliable route. Relevant differences in fluorescence intensities were observed upon comparing the galactose content in "healthy" IgG and galactose- deficient IgG. The reliability of the method was assessed by subjecting these IgG samples to the well-established *N*-glycan hydrolysis and subsequent analysis of the released galactose by capillary electrophoresis and high-performance liquid chromatography.
In the presence of a pathological targeted IgG, a peculiar decrease in fluorescence intensity has allowed the detection of galactose losses.

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P327
INDUCTION OF APOPTOSIS IN COLORECTAL CANCER CELLS BY INHIBITORS OF O-GLYCOSYLATION.

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Introduction
O-glycosylation is a common feature of many proteins involved in the regulation of cell growth pathways including apoptosis, but the mechanisms remain unclear. Inhibitors of O-glycosylation are limited to benzyl-*O*-N-acetyl-D-galactosamine, (benzyl-GalNAc) which acts as a competitive inhibitor leading to the intracellular accumulation of benzyl-oligosaccharides. There are no reports of the action of benzyl-GalNAc on cell growth. We have synthesized analogues of benzyl-GalNAc and examined their action on cell growth and apoptosis in human colorectal cancer cell lines
Experimental
Analogues of benzyl-GalNAc were synthesised. These included cyclohexyl- and phenylethyl-alpha-O-glycosides and azido-monosaccharide-alpha-O-glycosides of GalNAc. In addition the benzyl-alpha-C-glycoside and analogues were synthesized.
The inhibitors were tested with an established series of human colorectal cancer cell lines and the rate of growth, the proportion of shed cells with apoptotic properties, cell cycle phases, cell surface glycosylation and aglycone-oligosaccharide formation was measured. Cellular and subcellular glycosylation was analysed by confocal microscopy. Apoptosis was assessed using acridine orange/ethidium bromide staining, PARP cleavage and cell counts.
Results
The different cell lines showed variable susceptibility to the inhibitors, but all inhibitors tested resulted in smaller cell yields and induced apoptosis, with the C-glycosides being the most potent. A differential action on O-glycosylation was detected, evident in changes in subcellular localization in the various cell lines.
Conclusions
The results demonstrate that induction of apoptosis and the regulation of cell growth in colorectal cancer cells is dependent on O-glycosylation. The synthesis of benzyl-GalNAc analogues provides new tools to examine the mechanism of these events at the molecular level.

DECREASED α2,6 SIALYLATION IN COPD

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Many pathophysiological conditions involving inflammation were shown to be associated with aberrant protein glycosylation. Chronic obstructive pulmonary disease (COPD), a major global health problem with constantly increasing prevalence, also involves an abnormal inflammatory response. Although numerous glycoproteins were shown to play important roles in pathogenesis of COPD, the available information on glycosylation in this disease is extremely scarce. In our study encompassing 30 healthy (age 55.7±8.7; 10 smokers, 5 ex-smokers, 15 non smokers), and 29 individuals with diagnosed COPD (age 67.1± 7.5; 19 smokers, 5 ex-smokers, 5 no smokers) we examined the level of α2,6 sialylation of total serum proteins and transferrin. For the analysis of sialic acid content on serum transferrin we developed a novel lectin-based ELISA with immobilized antibodies. The level of α2,6 sialylation of total serum proteins was analyzed using slot blot method followed by lectin-chemiluminescence detection. The obtained results showed decrease in both, transferrin sialylation for 13% (P=0.0004), and total serum protein α2,6 sialylation for 15% (P=0.0019) in COPD patients compared to the controls. These findings offer a good basis for further glycobiological investigation of COPD and could be used for improvement of diagnostic approach in COPD. Furthermore, development of novel assay for transferrin sialylation represents a contribution to the studies of glycosylation changes in general.

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DECREASED α2,6 SIALYLATION IN COPD

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Many pathophysiological conditions involving inflammation were shown to be associated with aberrant protein glycosylation. Chronic obstructive pulmonary disease (COPD), a major global health problem with constantly increasing prevalence, also involves an abnormal inflammatory response. Although numerous glycoproteins were shown to play important roles in pathogenesis of COPD, the available information on glycosylation in this disease is extremely scarce. In our study encompassing 30 healthy (age 55.7±8.7; 10 smokers, 5 ex-smokers, 15 non smokers), and 29 individuals with diagnosed COPD (age 67.1± 7.5; 19 smokers, 5 ex-smokers, 5 no smokers) we examined the level of α2,6 sialylation of total serum proteins and transferrin. For the analysis of sialic acid content on serum transferrin we developed a novel lectin-based ELISA with immobilized antibodies. The level of α2,6 sialylation of total serum proteins was analyzed using slot blot method followed by lectin-chemiluminescence detection. The obtained results showed decrease in both, transferrin sialylation for 13% (P=0.0004), and total serum protein α2,6 sialylation for 15% (P=0.0019) in COPD patients compared to the controls. These findings offer a good basis for further glycobiological investigation of COPD and could be used for improvement of diagnostic approach in COPD. Furthermore, development of novel assay for transferrin sialylation represents a contribution to the studies of glycosylation changes in general.

P329
FUNCTIONAL CHARACTERIZATION OF HEREDITARY INCLUSION BODY MYOPATHY MUTANTS OF UDP-GLCNAC 2-EPIMERASE/MANNAC KINASE

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Hereditary inclusion body myopathy (HIBM) is a unique group of neuromuscular disorders characterized by adult-onset, slowly progressive distal and proximal muscle weakness, and a typical muscle pathology with cytoplasmic "rimmed vacuoles" and cytoplasmic or nuclear inclusions composed of tubular filaments. A single homozygous missense mutation was first identified in Persian and other Middle Eastern Jewish patients in the gene encoding UDP-GlcNAc 2-epimerase/ManNAc kinase (GNE), the key enzyme in the biosynthetic pathway of sialic acid (Eisenberg et al. (2001) Nat. Genet. 29, 83). Furthermore, different missense mutations in this same gene have been identified in quadriceps sparing HIBM cases diagnosed in several isolated families of non-Jewish origin.
In order to biochemically characterize the HIBM mutations of GNE, we generated mutations in both domains of the bifunctional enzyme by site-directed mutagenesis, and the resulting proteins were recombinantly expressed in insect cells by the baculovirus system. All mutant enzymes still displayed UDP-GlcNAc 2-epimerase as well as ManNAc kinase activities, but compared to the wild-type enzyme either one or both enzyme activities were reduced. Furthermore, models of the epimerase and the kinase domain of GNE were generated on the basis of known three-dimensional structures of related procaryotic UDP-GlcNAc 2-epimerases and sugar kinases, respectively. These models give a more detailed view into the structure of GNE, and suggest potenial effects of HIBM mutants, likely providing further indications of the molecular mechanism of the disease.

P330
DETERMINATION OF HETEROZYGOSITY OF *CDAN2* GENE IN TWO FAMILIES WITH CONGENITAL DYSERYTHROPOIETIC ANEMIA WITH A DIFFERENT GENETIC BACKGROUND.

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Introduction. Congenital dyserythropoietic anemias (CDAs) are a group of rare diseases characterized by ineffective erythropoiesis and the presence of bi- or multinucleated erythroblasts in the bone marrow. Inheritance of the disease in autosomal recessive. In the most common type CDA-II, the erythroblasts are bi-nucleated while circulating erythrocytes show a marked hypoglycosylation of erythrocyte anion exchanger (AE1). Other glycosylation abnormalities of CDA-II erythrocytes comprise hypoglycosylation and accumulation of polyglycosylceramides, as well as accumulation of Lc3Cer, nLc4Cer, and often of Gb4Cer (1). In addition, *O*-linked glycans of glycophorin A are partially unglycosylated. Previously, through analysis of all these biochemical markers we successfully determined heterozygous status of the disease carriers in a single family with CDA-II (2). The genetic background of the family was unknown.
Results. In this study, we examined two families: family 1 in which the disease gene (*CDAN2*) was mapped to chromosome 20q11.2 (3) and family 2 with a different genetic background (4). In spite of the different genetics, the glycosylation abnormalities in erythrocytes of four affected siblings from the two families were identical, and typical for CDA-II. This

enabled us to perform successfully determination of the heterozygous state in four *CDAN2* carriers (parents) from the two families.
Conclusions. Our results show that mutations in genes that localize to different chromosomes produce identical glycosylation abnormalities in CDA-II erythrocytes. This suggests that the genes in question regulate either different events on a common pathway - or cooperate in a single event - that is essential for normal cytokinesis of erythroblasts.
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P331
IDENTIFICATION AND FUNCTIONAL CHARACTERISATION OF A UDP-GLUCOSE PYROPHOSPHORYLASE FROM <I>LEISHMANIA MAJOR</I>

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Leishmania are protozoan parasites and cause diseases ranging from self-healing cutaneous lesions to lethal visceral forms. In Leishmania major, various glycoconjugates are essential for parasite virulence. They form a dense cell surface glycocalyx allowing the survival and proliferation of the parasite in very hostile environments. Thus, enzymes involved in the biosynthesis of the parasite glycocalyx provide interesting drug targets.
Essential for the biosynthesis of glycoconjugates is the metabolic activation of the monosaccharides as nucleotide sugars by pyrophosphorylases. We have isolated a UDP-glucose pyrophosphorylase (UDPGP) from Leishmania major that forms UDP-glucose from glucose-1-phosphate and UTP. The activation of glucose to UDP-glucose is crucial for the entry in biosynthetic pathways and is required for the synthesis of UDP-galactose, a major component of Leishmania glycoconjugates.
The activity of the UDPGP was proven by complementation studies of an E.coli galU mutant and by in vitro activity assays. Since it was postulated that the oligomeric state of UDPGPs has a critical impact upon catalysis, the oligomerisation status of the recombinant protein was determined. In contrast to UDPGPs from other organisms, the Leishmania major UDPGP exists as monomer exclusively and no higher order oligomers could be detected. These data are in agreement with the simple Michaelis-Menten-kinetics observed for all substrates.
Finally, the role of the UDPGP in the pathomechanism of Leishmania has been investigated by gene deletion. The obtained mutant demonstrated drastically reduced virulence in a mouse infection model, indicating the importance of this gene for pathogen development in the host.

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N-GLYCOSYLATION INHIBITION-INDUCED APOPTOSIS DURING ANGIOGENESIS IS INITIATED BY THE ER-RESIDENT CASPASE-12 AND MEDIATED BY CASPASE-7.

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Intrinsic apoptotic mechanisms arising from endoplasmic reticulum (ER) stress have been recently described. Short-term disruption of ER homeostasis by the N-glycosylation inhibitor tunicamycin (TM) has been shown to induce the unfolded protein response (UPR). But prolongued dysfunction of the ER machinery results in the induction of apoptosis in proliferating capillary endothelial cells. The ER-resident caspase-12 has been shown to initiate apoptotic signal transduction pathways triggered by ER-stress. Quantitative immunoblot and enzymatic analyses showed that short-term exposure to TM (<24 hrs) induced the UPR and an early activation of caspase-12, caspase-7 and a biphasic activation of caspase-9 through a cytochrome c-independent pathway. After prolongued ER-stress (>24 hrs) a cytochrome c-dependent pathway was activated with subsequent activation of the executioner caspase-3 and irreversible apoptotic induction, evidenced by Annexin-V binding and DNA "laddering" after 32 hours. Apoptotic induction was inhibited if TM was removed from culture medium within 24 hours, suggesting that the UPR was trying to restore ER homeostasis. But after 24 hours the UPR is no longer capable of sustaining normal ER function and the cell becomes committed to a programmed cell death. Early activation of caspase-7 might suggest that it is a substrate for caspase-12. Cell cycle analysis by flow cytometry in synchronized cultures indicated that all these events corresponded to the G1 phase, suggesting that protein N-glycosylation might play an important role in the G1/S transition checkpoint during angiogenesis. *Supported by MSEIP grant, USA Dept. of Education: P120A02008 and MBRS-RISE, NIH (USA): 1 R25 GM066250-01A1.*

P333
ALTERED CERAMIDE METABOLISM IS ASSOCIATED WITH PACLITAXEL RESISTANCE IN HUMAN OVARIAN CANCER CELLS

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Paclitaxel is an antimitotic agent used in the treatment of a number of major solid tumors, particularly in breast and ovarian cancer. Resistance to PTX has been observed in tumors and it represents a major impediment to the successful use of this agent in cancer treatment.
Paclitaxel treatment is able to induce apoptosis in a human ovarian carcinoma cell line, CABA I. Paclitaxel-induced apoptosis in CABA I cells was accompanied by an increase in the cellular ceramide levels and a decrease in the sphingomyelin levels, due to the activation of sphingomyelinases. The inhibition of acid sphingomyelinase reduced paclitaxel-induced apoptosis. Under the same experimental conditions, paclitaxel had no effects on ceramide and sphingomyelin levels in the stable paclitaxel-resistant ovarian carcinoma cell line, CABA-PTX.
The acquisition of the paclitaxel-resistant phenotype is accompanied by unique alterations in the complex sphingolipid pattern. In the resistant cell line, the levels of sphingomyelin and neutral glycosphingolipids were unchanged respect to the sensitive line. The ganglioside pattern of CABA I cells is more complex respect to CABA-PTX cells. Specifically, we found that the total ganglioside content in CABA-PTX cells was about half than in CABA I cells, and GM3 ganglioside is remarkably higher in resistant cell.
Taken together our findings indicate that i) ceramide, generated by acid sphingomyelinase, is involved in paclitaxel-induced apoptosis in ovarian carcinoma cells and paclitaxel-resistant cells are characterized by the lack of ceramide increase upon drug treatment; ii) paclitaxel resistance might be correlated with alteration of ceramide metabolic patterns affecting specifically the cell ganglioside composition.

P334
DIFFERENTIAL GLYCOSYLATION AND SULFATION IN AIRWAY MUCINS OBSERVED IN CYSTIC FIBROSIS

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Cystic Fibrosis (CF) is the most lethal genetic disease in Caucasians and is characterized by the production of excessive amounts of viscous mucus in the airways of the patients leading to airway obstruction and bacterial infections, especially with Pseudomonas aeruginosa (PA). Our studies and others have shown that PA binds preferentially to CF airway mucins than to non-CF mucins. To date, the molecular basis of increased PA binding to CF mucins is poorly understood. To address this question, we examined the O-glycan structure of purified mucins isolated from mucus secretions of CF and normal individuals. CF mucins had higher sugar content and more O-glycans per mg as compared to normal mucins. Also, there was two fold increase in sialylation and sulfation in CF compared to normal mucins. O-glycans were released from CF and normal mucins and derivatized with 2-aminobenzamide, separated by ion-exchange chromatography and quantified by fluorescence. HPLC profiles of O-glycans showed differences between samples isolated from CF and normal patients. Both neutral and acidic O-glycan fractions were collected. Glycan compositions were defined by MALDI-TOF, mass spectrometry. In CF mucins eight distinct neutral O-glycans were identified . In addition, CF mucins contained a higher proportion of sialylated and sulfated O-glycans compared to normal mucins. While both, CF and normal mucus contained GlcNAc-6-Sulfate, Gal-6-Sulfate and Gal-3-Sulpfate, CF mucins had higher amounts of the 6-sulfated species. These profound structural differences in mucin glycosylation in CF may explain increased pathogenesis by PA. Supported, in part, by NIH grant : HL065509.

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TRANSCRIPTIONAL ACTIVATION OF THE HUMAN β-1,4-GALACTOSYLTRANSFERASE V GENE BY TRANSCRIPTION FACTOR ETS-1

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β-1,4-Galactosyltransferase (β-1,4-GalT) V is a ubiquitously expressed enzyme that can effectively galactosylate the GlcNAcβ1-6Man group of the highly branched N-linked oligosaccharides. Upon malignant transformation of cells, the expression of the β-1,4-GalT V gene increases in accordance with the increase in the amounts of highly branched N-linked oligosaccharides. We found that the expression of the human β-1,4-GalT V gene increases by Ets-1 as well as Sp1. Luciferase assay showed that the region between nucleotide positions -116 and +22 relative to the transcriptional start site of the β-1,4-GalT V gene is involved in the activation of its promoter by Ets-1. Although the promoter region -116/+22 of the β-1,4-GalT V gene contained one Ets-1-binding site, which overlapped with the Sp1-binding site, Ets-1 but not Sp1 failed to bind to this promoter region as revealed by the

electrophoretic mobility shift assay. Since the promoter region of the human Sp1 gene contains two Ets-1-binding sites, whether or not the expression of the Sp1 gene is regulated directly by Ets-1 was examined. The results showed that upon introduction of the ets-1 gene into A549 human lung carcinoma cells, which show low expression of Ets-1, not only the gene expression but also the promoter activity of Sp1 increased by 3- to 4-fold, but both decreased dramatically by the transfection of its dominant-negative mutant gene into HepG2 human hepatocarcinoma cells, which show high expression of Ets-1. These results indicate that Ets-1 enhances the expression of the β-1,4-GalT V gene through activation of the Sp1 gene.

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ASPERGILLUS FUMIGATUS CONIDIAL SURFACE SIALIC ACIDS: A TARGET FOR ANTI-FUNGAL DRUGS?

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Introduction:
Aspergillus fumigatus is the most prevalent airborne fungal pathogen in developed countries, and in immuno-compromised patients causes the usually fatal disease, invasive aspergillosis (IA). *A. fumigatus* infection begins with the inhalation of conidia, which adhere to and germinate in the lung. Recent studies indicate that binding of conidia to lung extracellular matrix (ECM) proteins may be mediated by sialic acids (Sia) [1,2].
Experimental approach:
The binding of *A. fumigatus* conidia to a number of ECM proteins was evaluated using a microtiter plate adherence assay as described [3]. The capacity of Sia and a number of novel Sia-based compounds to interfere with this binding was also evaluated.
Results:
A. fumigatus conidia were able to bind fibronectin, fibrinogen, laminin and collagen type IV in a concentration dependent manner. Binding was inhibited by free *N*-acetylneuraminic acid (100% at 10 mM) and fetuin (100% at 0.5 mg/ml), as well as by a number of novel Sia-based compounds. However, no inhibition was noted for *N*-acetyl-2-deoxy-2,3-didehydro-neuraminic acid at 10 mM.
Conclusion:
Exogenously added Sia and novel Sia-based compounds were found to inhibit the binding of *A. fumigatus* conidia to ECM proteins. This suggests that conidial binding to ECM proteins on epithelial cells of the lung may represent a target for novel anti-fungal agents based on Sia.

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P337
CONNECTIONS BETWEEN APOPTOSIS AND SIALIC ACID METABOLISM: APPLICATION TO NEW CANCER THERAPIES.

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Aberrant sialic acid biosynthesis is a distinguishing feature of many types of cancer. Strategies to employ *N*-acetylmannosamine (ManNAc) analogues as molecular tools to ameliorate sialic acid-related metabolic abnormalities and thereby create a new class of anti-cancer drugs are under development in several laboratories. So far, these efforts have largely focused on ManNAc analogues that successfully transit the sialic acid pathway and appear on the cell surface as the corresponding non-natural sialosides. The surface presentation of non-natural sialic acids alters the adhesive properties – and potentially the metastatic potential – of a cancer cell, provides epitopes recognized by immunosurveillance mechanisms, or supplies 'chemical handles' for the delivery of diagnostic or cytotoxic agents. Recent results from our laboratory have established that ManNAc analogues also have intracellular effects that accompany the cell surface display of sialic acids; of particular note is the potent induction of apoptosis in cancer cells by certain of these sugars. This finding corresponds to a body of experimental evidence compiled over the past few years that has firmly established sialic acid as an important molecular player in several aspects of the instigation and execution of apoptosis both via sialoglycoproteins, such as Fas, and through glycosphingolipids that include the gangliosides GM3 and GD3. In this talk, the molecular mechanisms putatively responsible for the apoptotic properties of ManNAc analogs will be presented along with strategies designed to selectively direct these effects to malignant cells, which is a necessary step if these compounds are to become viable candidates as anti-cancer drugs.

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GANGLIOSIDES ARE IMPORTANT FOR FORMATION AND MAINTENANCE OF ION CHANNEL CLUSTERS IN MYELINATED NERVE FIBERS

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Gangliosides are abundantly present in the nervous systems, whereas their neurobiological functions are not fully understood. To elucidate the physiological role of gangliosides in saltatory conduction along the myelinated nerve fibers, we examined mice lacking complex gangliosides produced by disruption of the GM2/GD2 synthase gene. Motor nerve conduction velocities in sciatic nerves significantly reduced in mutant mice. Compound muscle action potential amplitudes significantly decreased in 48-week-old mutant mice, whereas those of 12-weeks-old mutant mice were preserved. Actively clustering ion channels at regularly spaced gaps in the myelin sheath called nodes of Ranvier are required for rapid conduction along myelinated nerve fibers. Structures around the node in ventral roots were examined by immunohistochemical study. The staining of potassium channel was aberrantly present at paranode. The length of sodium channel staining at node was significantly longer. Abnormal protrusion of sodium and potassium channel staining were also frequently noted. These findings suggest disrupted nodal and paranodal structures in the mutant mice. Electron micrographs revealed that myelin loops occasionally failed to contact the axolemma and abnormal protrusion of nodal and paranodal axolemma were frequently seen. The paranodal potassium channel was observed more frequently in the mutant mice than wild type during the developmental period, and became prominent with age. In the mutant mice, disrupted nodes and paranodes may cause alteration of ion channel clusters and consequent nerve conduction slowing. Gangliosides are important for formation and maintenance of structures and ion channel clusters around the node of Ranvier in the myelinated nerve fibers.

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