# Abstracts of the Joint Meeting of the Society for Complex Carbohydrates and the Mid-West Connective Tissue Workshop.

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# MINISYMPOSIA

**1**

THE BINDING OF THE LEWIS B TETRASACCHARIDE BY THE LECTIN IV OF *GRIFFONIA SIMPLICIFOLIA*. R.U. Lemieux,* U. Spohr,* P. Nikrad,* J. Pearlstone,** M.R. Carpenter,** L.B. Smillie,** M. Vandonselaar,† J.W. Quail,† L. Prasad,† L.T.J. Delbaere,† *Department of Chemistry and **MRC Group in Protein Structure and Function, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada, T6G 2G2. †Department of Biochemistry, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N OWO.

The lectin [Shibata, Goldstein and Baker (1982)] is now known to be a dimeric metalloglycoprotein ($Ca^{++}$, $Mn^{++}$ and 243 amino acids in each subunit). Its affinity ($K_{Assoc.} = 4.4 \times 10^4\ M^{-1}$ at 298°) for $Le^b$-OMe ($\alpha$LFuc(1→2)$\beta$DGal(1→3)[$\alpha$LFuc-(1→4)]-$\beta$DGlcNAc-OMe has been studied extensively by probing the combining site with chemically modified structures (Spohr and Lemieux, *Carbohydr. Res.*, 1988, **174**, 211 and references therein.) The amino acid sequence was established in Smillie's laboratory and the crystal structures of both the lectin and its complex with $Le^b$-OMe in Delbaere's laboratory. The glycoconjugation is at ASN-5 and ASN-18 of one subunit but at only ASN-18 of the other.

The structure of the complex proved to be in good general agreement with expectations based on the results of the probing data and the changes in thermodynamic parameters with changes in the structure of the epitope. The key polar interaction with SER-49, ASP-89 and ASN-135 is at the bottom of a shallow well with the walls lined by the aromatic groups of PHE-108, TYR-105, TYR-233, TRP-133 and TRP-138. Thus, the nonpolar interactions between the aromatic groups with the nonpolar regions of the tetrasaccharide must provide a shield from exchanges with water that would decompose the complex. The key polar interaction involves hydrogen bonding of the carboxylate group of ASP-89 with OH-3b and (through a water molecule) with OH-4b of the $\beta$DGal b-unit. Also, the hydroxyl of SER-49 accepts a proton from OH-4c of the $\alpha$LFuc(1→4) c-unit. ASN-135 and TYR-105 also become involved in the hydrogen-bonds with OH-3c and SER-49, respectively. The specificity of the binding is considered to be mainly related to the polar interaction with the nonpolar interactions being mainly concerned with the stability of the complex.

**2**

SPECIFICITY IN OLIGOSACCHARIDE-PROTEIN INTERACTION. J.P. Carver. Department of Medical Genetics and Medical Biophysics, University of Toronto, Ontario M5S 1A8, Canada.

**3**

COMPARISON OF THE REFINED HIGH RESOLUTION CRYSTAL STRUCTURES OF WHEAT GERM ISOLECTINS 1 AND 2 AND THEIR COMPLEXES WITH N-ACETYLNEURAMINYL LACTOSE. Christine S. Wright, Department of Biochemistry and Molecular Biophysics, Medical College of Virginia, Richmond, Va. 23298.

Wheat germ agglutinin(WGA) is a member of a group of evolutionarily conserved lectins from the gramineae family that share highly stable disulfide-rich structures with a four-fold sequence repeat and specificity for N-acetylated saccharides (N-acetylglucosamine, N-acetylneuraminic acid). X-ray diffraction studies have been carried out on isomorphous crystals of two of the three genetic variants characterized in polyploid wheat. These isolectins (WGA1 and WGA2) differ at 5 sequence positions and exhibit different electrophoretic mobilities and affinities for N-acetylneuraminyl lactose (NeuNAc-Gal-Glc)(Kronis & Carver, Biochemistry 21, 3052 (1982). Of the two types of sugar binding sites (four total) present in the subunit/subunit interface of the WGA dimer, only one, termed 'primary' or P-site, was found to be accessible in the crystal to sialyl-saccharides, and could thus far be characterized in detail.

High resolution refinement was carried out at 2.0Å and 1.8Å resolution on WGA1 and WGA2, respectively, as well as on their corresponding neuraminyl lactose complexes at 2.2Å resolution (R-factors ranging from 15.6% to 17.9%). This has allowed more accurate positioning of the trisaccharide and a detailed comparison of its binding mode in the two isolectins at the P-site. It has also made it possible to assess the extent of mobility of crucial amino acid residues and determine the positions of bound water. Superposition of the two trisaccharide complexes indicates a close match only of the terminal NeuNAc, bound by 4 H-bonds and 18-20 van derWaals' contacts with 3 aromatic sidechains. Poor alignment, high atomic temperature factors and poor electron density are observed for the remaining Gal-Glc portion. Two water molecules are displaced and one repositions to solvate the C4-OH of NeuNAc. The presence of a Tyr in WGA1 at residue 66 as compared to His in WGA2 provides an additional H-bond and more extensive van der Waals' contact for stabilization of the WGA1 complex. Conformational changes of several residues in the binding site and differences in mobility of the native structures versus those of the complexes will be discussed.

**4**

COMPARISON OF TWO GAL-SPECIFIC LECTINS OF ANIMAL ORIGIN. Y.C. Lee and R.T. Lee. Department of Biology, THE JOHNS HOPKINS UNIVERSITY, Baltimore, MD 21218

Mammalian hepatic lectins and galaptins both recognize galactose, but they belong to different lectin families. Inhibition studies indicated that Gal is bound much more tightly to the hepatic lectin than to galaptin. However, lactose had very similar affinity to both lectins. The penultimate β-1,4-linked Glc in Lac is only nonspecifically interacting with the hepatic lectin, since the affinity of Lac was similar to many different kinds of β-galactosides. On the other hand, Lac binds to galaptin ca. 200-fold tighter than the average β-O-galactosides, indicating a specific interaction of Glc residue with galaptin. This specific interaction appears to occur mainly with the 3-OH group and to some extent with the equatorial 2-substituent of Lac. When LacNAc, the best disaccharide inhibitor, was converted to 3-deoxy-LacNAc, the affinity decreased 50-fold, indicating that 3-OH not only has to be unsubstituted, but also has to be present. The equatorial 2-substituent can enhance the affinity by as much as 5-fold, and the order of affinity enhancement is $NHAc > CH_2OH > OH$.

The most striking difference between the two lectins is their behavior towards the so-called cluster ligands. Gal-terminated bi- and tri-antennary structures can possess $10^3$ and $10^5$-fold stronger binding affinity, respectively, to the hepatic lectin than a monoantennary structure. However, the inhibition assay with the same clustered lactosides and asialoglycopeptides of complex type showed that the affinity enhancement was no more than 3-fold for galaptin. The most prevalent triantennary structure in the desialylated serum glycoproteins has $I_{50}$ of 7 nM for the hepatic lectin, and 46 μM for galaptin (6600-fold higher), and asialoorosomucoid bound nearly 10,000-fold tighter to the hepatic lectin than to galaptin.

5

FORMATION OF CRYSTALLINE LATTICES BETWEEN CELL SURFACE OLIGOSACCHARIDES AND LECTINS. AN ELECTRON MICROSCOPIC AND X-RAY DIFFRACTION STUDY. C.F. Brewer[1], J. Fant[2], M.I. Khan[1], L. Bhattacharyya[1] and L. Makowski[3].
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Biantennary complex type oligosaccharides containing the $Le^x$ (or type 2 chain of $Le^a$) antigenic determinant (Galβ(1-4)[Fucα(1-3)]GlcNAc) at the nonreducing terminii and an agalacto analog bind and precipitate as divalent ligands with the tetrameric L-fucose specific isolectins A and C from Lotus tetragonolobus (LTL-A and LTL-C, respectively). Negative stain and freeze fracture electron microscopic (EM) images of the precipitates of LTL-A with the oligosaccharide show a unique pattern for each indicating long-range order in the precipitates characteristic of crystalline lattices. However, the precipitates with LTL-C failed to show any pattern. EM studies with the precipitates formed between soybean agglutinin and bi- or tetraantennary oligosaccharides show similar high degree of organization and long-range order. The results demonstrate that the precipitates of certain bi- and tetraantennary complex type oligosaccharides with the lectins form highly organized cross-linked structures, and that each oligosaccharide forms a unique homogeneous lattice with a given protein. The latter conclusion is consistent with our recent finding using quantitative precipitation analysis that a variety of oligomannose and bisected hybrid type glycopeptides form homogeneous lattices with concanavalin A from a binary mixture of the carbohydrates (Bhattacharyya et al. (1988) Biochemistry 27, 8762). X-ray diffraction analysis confirms the crystalline nature of the lectin-carbohydrate precipitates. Furthermore, the lattice geometries of the lectin cross-linked complexes can be analyzed and information obtained on the conformation of the bound oligosaccharides. The results indicate that the specificity of interactions of multivalent N-linked oligosaccharides with multivalent lectins is greater in their cross-linked states than in their soluble complexes, and that isolectins may possess different precipitating activities and form different cross-linked lattices with N-linked oligosaccharides. These findings may relate to the possible biological roles of lectins and N-linked carbohydrates as receptors.

6

GENETIC REGULATION OF GLYCOLIPID EXPRESSION.

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The polymorphic expression of glycolipids and the changes of carbohydrate structures of glycolipids during embryogenesis, differentiation and malignant transformation are well described. However, molecular mechanisms producing such expression and changes are not well understood and we assume that genetic and epigenetic regulations are involved. One of our long-term projects is to elucidate the molecular mechanism of the genetic regulation.

We had found the polymorphic expression of hematoside or GM3 in the erythrocytes of dogs and reported that an autosomal gene is involved in the expression of GM3(NeuGc). The gene is suggested to regulate the activity of enzyme, which adds oxygen to NeuAc of either CMP-NeuAc or GM3(NeuAc). It is of interest that dogs of oriental breeds have GM3(NeuGc) but none of European breeds, as far as we examined, has GM3(NeuGc).

We, then, selected mice as an experimental animal to study the mechanism of genetic regulation in detail. We have been able to define 5 genes, which are involved in the expression of carbohydrate structures of glycolipids and act in a tissue specific manner. In this paper, 3 genes out of the 5 will be discussed. Ggm-1, Ggm-2 and Gsl-5 control the expressions of GM1(NeuGc), GM2(NeuGc), both in the liver and erythrocytes, and Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-3)GalNAcβ1-3Gb3Cer in the kidney, respectively. Ggm-1 regulates the activity of galactosyltransferase to synthesize GM1(NeuGc) and is located 1 cM centromeric to H-2K on chromosome 17. Ggm-2 regulates N-acetylgalactosaminyltransferase activity for GM2(NeuGc) synthesis. Gsl-5 controls N-acetylglucosaminyltransferase activity for the synthsis of GlcNAcβ1-6(Galβ1-3)Gb4Cer and is located on chromosome 19. Then, we need to know their primary products and genetic information.

## 7

TWO NOVEL GLYCOCONJUGATE-CLEAVING ENZYMES FROM THE LEECH. Y.-T. Li. Department of Biochemistry, Tulane University School of Medicine, New Orleans, La. 70112

Leeches consume animal blood, which is rich in glycoconjugates. Based on this fact, we reasoned that leeches should be able to catabolize glycoconjugates. Indeed, leeches are very rich in glycosidases, and herewith we report the presence of two novel glycoconjugate-cleaving enzymes in a North American leech, Macrobdella decora.

A Glycolipid Glycan-Detaching Enzyme - Ceramide-glycanase(CG) is an enzyme that releases the intact glycan chain from various glycosphingolipids (GSLs) by cleaving the linkage between the ceramide and the glycan chain. We have devised a simple method for the purification of CG from M. decora. The relative rates for the hydrolysis of the common GSLs by CG are: $Gg_3Cer > Gg_4Cer > GM2 > GM1 > Gb_3Cer > Gb_5Cer > GD1a > nLc_4Cer > GM3 > LacCer-{}^3II\text{-}SO_4 > nLc_3Cer > Gb_4Cer > GT > LacCer$. In addition, CG also detached glycan chains from lyso-GSLs, lactosyl-diglyceride and alkyl-lactosides. CG was found to recognize the hydrophobic portion as well as the sugar moiety of the glycolipid substrates. The detachment of glycan chains from glycolipids greatly facilitates their structural analysis. Among the various animal tissues examined, CG was found only in annelids.

A Novel Sialic Acid-Cleaving Enzyme- Leeches contain two sialic acid-cleaving enzymes, an ordinary sialidase and a novel sialic acid-cleaving enzyme (NE). Instead of NeuAc, NE released a novel NeuAc-derivative (I) from 4-methylumbelliferyl-NeuAc(MU-NeuAc), and NeuAc-containing glycoproteins and GSLs. We have partially purified NE from M. decora. We have also isolated I released from MU-NeuAc and whale nasal keratan sulfate. I was found to be resistant to NeuAc-aldolase. It also did not give color by periodate-thiobarbituric acid reagent. By using chemical analysis, FAB-MS and NMR spectroscopy, the structure of I was tentatively identified as NeuAc-2,7-inner glycoside. I was also released from $\alpha_1$-acid glycoprotein, fetuin, neuramin-lactose, GM3 and GD1a, but was not produced from free NeuAc. NMR analysis showed that instead of the 1C conformation as in the case of NeuAc, the pyranose ring of I was in the C1 conformation which makes the formation of the 2,7-inner glycoside possible. We propose to call I, "tulanic acid" and NE, "sialidase-T". (Supported by NSF DMB 8617033 and NIH NS 09626).

## 8

REGULATION OF GALACTOSYL- AND FUCOSYLTRANSFERASES OF TUMOR CELL ORIGIN M. Basu, S.-A. Weng, K.K. Das and B.-Z. Zhang, Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556

The presence of asialolactosaminyl groups (NeuAc$\alpha$2-3Gal$\beta$1-4GlcNAc-) and sialo-$Le^x$ (NeuAc$\alpha$2-3Gal$\beta$1-4(Fuc$\alpha$1-3)GlcNAc)- appears to be ubiquitous on animal tumor cell surfaces. The expression of these glycosphingolipids (GSLs) on cell surfaces may be due to the differential expression of specific glycosyltransferase (e.g., GalT-4, SAT-3 and FucT-3) genes or to post-translational regulation of these activities.

Recently, we were able to solubilize both GalT-4 (UDP-Gal:Lc3 $\beta$1-4GalT) and FucT-3 (GDP-Fuc:LM1 $\alpha$1-3FucT) from human colon carcinoma Colo 205 cells. GalT-4 has also been solubilized (Triton CF-54) from mouse T-lymphoma (P-1798) and purified (35,000-fold) by ion-exchange and $\alpha$-lactalbumin affinity column chromatography. A systematic study with phospholipid liposomes revealed that both phosphatidyl ethanolamine and phosphatidyl choline stabilize and stimulate (2-fold) the purified GalT-4 activity from P-1798, perhaps by interacting with the hydrophobic domain. The GalT-4 activities from both P-1798 and Colo-205 were inhibited (50%) in the presence of 0.4 mg/ml D-sphingosine. However, an initial stimulation (2-fold) was observed with Colo-205 GalT-4 only in the presence of 0.15 mg/ml D-sphingosine. Fifty percent inhibition of Colo 205 FucT-3 activity was obtained only in the presence of higher concentrations of D-sphingosine (3 to 4 mM). The discovery of ceramide glycanase (CGase) in animal tissues (leech, earthworm and rabbit mammary gland) and ceramidase (rat brain) raises the question of the role of sphingosine in the regulation of glycolipid:glycosyltransferases.
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**9**

BIOSYNTHESIS OF LINEAGE-SPECIFIC GLYCOSPHINGOLIPID ANTIGENS BY HUMAN LEUKOCYTES. Bruce A. Macher and Cheryl L. M. Stults. Department of Chemistry and Biochemistry, San Francisco State University, San Francisco, CA 94132

Our work has focused on the analysis of glycosphingolipids expressed by human leukocytes with the goal of determining whether normal leukocytes express lineage-specific glycosphingolipids. We have established that lineage-specific neutral glycosphingolipids are expressed by normal leukocytes, but only by cells which have reached a certain stage of differentiation. Specifically we have shown that mature myeloid cells (granulocytes) express only neolacto neutral glycosphingolipids, whereas mature lymphocytes exclusively express globo compounds. Our next goal is to correlate the activity of the glycosyltransferases responsible for the synthesis of these compounds with their level of expression during leukocyte differentiation. To reach this goal, we have developed a new assay system to measure the activity of glycosyltransferases. In this system, product formation is specifically detected and quantified using a carbohydrate-specific antibody in conjunction with ELISA methodology. It has been determined that this assay can be used with detergent solubilized microsomal preparations of glycosyltransferases from human myeloid leukemia cells to measure the synthesis of the lineage-specific, myeloid antigen $nLc_4Cer$ from $Lc_3Cer$ and UDP-Gal via a β1-4 galactosyltransferase. Conditions are being establish to measure the lymphoid, lineage-specific antigen $Gb_3Cer$ and another myeloid specific ganglioside designated VIM-2, which we have recently structurally characterized. The latter compound was detected with a myeloid-specific antibody and was found to have the following structural epitope carried by a deca and dodeca ganglioside:

$$\begin{array}{l} \text{NeuAc}\alpha 2\rightarrow 3\text{Gal}\beta 1\rightarrow 4\text{GlcNAc}\beta 1\rightarrow 3\text{Gal}\beta 1\rightarrow 4\text{GlcNAc}\beta 1- \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 3 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \uparrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 1\alpha\text{Fuc} \end{array}$$

This antigen can be distinguished immunochemically from its structural isomer, Sialyl-SSEA-1, which is another glycosphingolipid antigen expressed by human myeloid cells. Thus, our new assay should allow us to specifically measure the synthesis of isomeric carbohydrate antigens. These studies were supported in part by NIH grant CA 32826.

**10**

ANALYSIS OF GLYCOSAMINOGLYCAN-DERIVED OLIGOSACCHARIDES USING REVERSED PHASE ION-PAIRING AND ION-EXCHANGE CHROMATOGRAPHY WITH SUPPRESSED CONDUCTIVITY DETECTION. R.J. Linhardt[1], K.N. Gu[1], D. Loganathan[1], and S.R. Carter[2] [1]Division of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa City, Iowa 52242 and [2]Dionex Corporation, Sunnyvale, CA 94088-3603

HPLC has been widely used for the structural determination and analysis of complex glycosaminoglycans (GAGs). Enzymatic depolymerization of uronic acid containing GAGs, including heparin, heparan sulfate, dermatan and chondroitin sulfates, using polysaccharide lyases, affords oligosaccharides having an unsaturated uronic acid residue, at their non-reducing terminus which can be conveniently detected by measuring absorbance at 232 nm. Deaminative cleavage of N-sulfated GAGs results in ring contraction and breakage of the adjacent glycosidic linkage affording an anhydromannose residue which can be radiolabelled using $NaB^3H_4$ or tagged with a fluorescent label to obtain detectable derivatives. GAGs which contain neither uronic acid nor N-sulfation such as keratan sulfate can be hydrolyzed chemically using acid or enzymatically using keratan hydrolase, but the resulting oligosaccharides are not easily detected. A simple HPLC based analysis capable of separating and detecting any GAG-derived oligosaccharide without derivitization would therefore be extremely useful.

Conductivity detection has been established in the analysis of inorganic and organic ions and hence should be applicable to the analysis of the carboxylate and sulfate salts common to all GAGs. Suppressed conductivity detection represents a relatively recent technological improvement making it possible to substantially increase detector sensitivity by reducing eluant conductivity. This approach also permits the on-line removal (prior to detection) of ion-pairing reagents often useful in separating highly polar molecules such as GAG-derived acidic oligosaccharides.

Reverse-phase ion-pairing (RPIP)-HPLC and ion-exchange chromatography (IC-HPLC) with suppressed conductivity detection (into the picomole range) was used to analyze oligosaccharides derived from GAGs.

11

## CELL-FREE BIOSYNTHESIS OF THE GLYCOSYL-PHOSPHATIDYLINOSITOL MEMBRANE ANCHOR OF THE TRYPANOSOME VARIANT SURFACE GLYCOPROTEIN

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Numerous important proteins are anchored to the membrane by glycosyl-phosphatidylinositol moieties (GPI-anchors) at their carboxyl termini. The best studied GPI-anchor is the *dimyristyl*-GPI-anchor found on the variant surface glycoprotein (VSG) of African trypanosomes. Recently, we have described a cell-free system in trypanosomes that is ideally suited for elucidating the biosynthetic pathway of GPI-anchor assembly (***Cell* 56, 793-800 (1989) & *J. Biol. Chem.* 264, 11168-11173 (1989)**). Based upon pulse-chase experiments with radiolabeled sugar nucleotides and various non-labeled precursors, and product characterization by gel filtration, thin-layer chromatography, glycosidase and lipase sensitivities, and by DIONEX HPLC, we have established the basic pathway for GPI-anchor assembly to be as follows: In the first-committed step, GlcNAc is donated from UDP-GlcNAc to endogenous phosphatidylinositol(s), which do not contain myristate. Purification of this GlcNAc transferase is underway. The GlcNAc-PI is then rapidly deacetylated to form GlcN-PI. GlcN-PI is then elongated by the addition of 3 mannosyl residues, one or more of which are donated by a mannosyl phophoryldolichol-like lipid intermediate. Ethanolamine-phosphate is subsequently added from an unknown donor. $EtNH_2$-P-Man3GlcN-PI then undergoes a series of novel fatty acid remodeling reactions, involving a GPI-specific phospholipase $A_2$, that yields the completed anchor precursor, which is a *dimyristyl*-GPI and serves as the anchor donor to nascent VSG. (Supported by NIH AI21334 and the MacArthur Fdn. Abstract sponsored by DIONEX)

12

## PURIFICATION AND CHARACTERIZATION OF RECOMBINANT PNGase F EXPRESSED IN ESCHERICHIA COLI

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Peptide-$N^4$-(N-acetyl-β-glucosaminyl) asparagine amidase F (PNGase F; EC 3.5.1.52) from Flavobacterium meningosepticum (ATCC 33958) is a useful enzyme for cleaving the Asn-linked oligosaccharides of glycoproteins. Crude preparations of PNGase F from the culture medium of F. meningosepticum fermentations contain endoglycosidase F (Endo F) activity and extensive purification is required to remove this contaminant from PNGase F preparations.

We have cloned the PNGase gene in E. coli by standard techniques using oligonucleotide probes designed to hybridize to a portion of the N-terminal coding region of mature PNGase F. DNA sequence analysis of the PNGase F gene insert identified a 25 amino acid coding region that corresponded to the N-terminal sequence obtained by protein sequence analysis of mature PNGase F purified from F. meningosepticum.

Recombinant PNGase F has been purified from extracts of E. coli containing the PNGase F gene. The purified recombinant enzyme released the Asn-linked oligosaccharides from ribonuclease B, $\alpha_1$-acid glycoprotein, and transferrin indicating that recombinant PNGase F has a substrate specificity similar to the native enzyme.

13

## HPLC STRATEGY AND TECHNIQUES USED IN THE CHARACTERIZATION OF OLIGOSACCHARIDES DERIVED FROM GLYCOPROTEINS.

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One of the most challenging applications using High Performance Liquid Chromatography is the analysis and purification of oligosaccharides derived from glycoproteins. There have been numerous reports in the literature describing the analysis of mono-, di- and oligosaccharides using a variety of column chemistries and by far the most popular are the amino-bonded phases. Recent advances in column technology, however, have provided carbohydrate researchers with new stable polymeric packing materials which give excellent resolution in both the analytical and preparative scales. HPLC techniques and an analytical strategy for the use of multi-column chemistries will be presented, to help address the needs of biotechnology companies and researchers to analyse and purify complex carbohydrates derived from glycoproteins. Included are methods used to gain information about site-specific glycosylation, by employing peptide mapping and amino analysis, as well as recent developments in carbohydrate profiling and purification prior to structure elucidation.

14

## REVIEW OF STRATEGIES FOR THE STRUCTURAL ELUCIDATION OF COMPLEX CARBOHYDRATES

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The complete elucidation of complex carbohydrate structures requires the simultaneous determination of several structural parameters. Spectroscopic techniques such as soft ionization mass spectrometry and high resolution nuclear magnetic resonance (NMR) can provide, when combined in special cases with enzymatic or immunological procedures, complementary information with minimal loss of valuable material and time: the number of sugar residues can be determined using MS and the interpretation of 1D-1H, 1D- or 2D-1H-correlated NMR spectra; the identification of the sugar residues, including their anomeric configuration, is possible using the determination of vicinal coupling constants, and chemical shifts, as well as immunological or enzymatic methods. Sequence and linkage patterns can be deduced from a combination of MS and NMR spectral data, with NOE measurements and any inter-residue long range couplings providing additional information. The 3-D arrangement of carbohydrate residues in space, whether in glycolipids or glycoproteins, occurring in free solution or bound to membrane surfaces, is still a matter for debate; energy minimization calculations combined with experimental data from the measurement of NOE's, cross relaxation rates, coupling constants, etc. especially when obtained in organic solvents have to be interpreted with caution particularly if one wishes to extrapolate to the situation occurring in vivo.

15

Structural Analysis of Bacterial O–Antigens;
Integration of High Resolution NMR and Classical Approaches.

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Structural analysis of bacterial O–antigens that show serological cross–reaction with A and M polysaccharide antigens of *Brucella* has provided a rich source of challenging structures. Even at 500MHz field strength the $^{1}H$ NMR spectra of many of these polysaccharides defy complete assignment. When this occurs structure elucidation by NMR techniques alone becomes a highly questionable objective, even if sufficient spectrometer time is available. In these cases $^{13}C$ NMR spectroscopy is generally helpful and a judicious combination of $^{1}H$ and $^{13}C$ NMR experiments often represent the optimum use of material and spectrometer time.

The structural studies discussed will illustrate the use of one and two dimensional NMR methods in tandem with classical approaches such as methylation analysis and specific degradation to oligosaccharides. The inter–residue nOe has been used extensively to determine monosaccharide sequence in the antigen repeating unit. Use of potential energy calculations based upon the HSEA algorithm to rank the relative magnitude of nOe when these measurements are used for sequence determination will be discussed. Combination of conformational predictions with nOe measurements were used in some instances to establish the anomeric as well as the absolute configuration of one hexopyranosyl residue, provided this information was known for the other hexose of a disaccharide fragment. The use of $^{13}C$ spectra to complement $^{1}H$ NMR data will be illustrated for several examples, especially those polymers which gave complex and tightly coupled proton spin systems.

16

CHEMICAL ANALYSIS OF COMPLEX CARBOHYDRATES BY THE REDUCTIVE-CLEAVAGE METHOD. G. R. Gray, Department of Chemistry, University of Minnesota, Minneapolis 55455

The determination of structure of complex carbohydrates is important in many areas of biomedical investigation because of the need to understand the molecular details of a wide variety of immunological and cellular recognition phenomena. Because of the compositional and structural complexities encountered in these polymers, many chemical as well as spectroscopic procedures for structural analysis have been developed. Unfortunately, most of these methods suffer from lack of sensitivity or generality, requiring the analyst to use several techniques for complete structural analysis.

In an attempt to simplify the structural analysis of complex carbohydrates, we have developed (1) a new, general procedure which we refer to as the "Reductive Cleavage Method." This method is based upon the regiospecific reductive cleavage of all glycosidic linkages in a fully methylated saccharide. The products, partially methylated anhydroalditols, are either acetylated and analyzed by GLC/MS, or benzoylated and analyzed by HPLC and $^{1}H$ NMR spectroscopy (2). Thus, in one experiment, the composition of a saccharide can be determined as well as the ring form(s) and position(s) of linkage of each of its residues. The sequence of the saccharide and the anomeric configurations of its component residues are derived in a separate experiment where reductive cleavage is accomplished under selective conditions; the oligomeric products are then benzoylated, separated by HPLC, and analyzed by $^{1}H$ NMR spectroscopy and mass spectrometry (2), or alternatively, are acetylated and analyzed by GLC/MS (3).

The scope and generality of this method will be illustrated using complex carbohydrates of widely differing compositions and structures.

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17

**SULFATED N-LINKED OLIGOSACCHARIDES IN DICTYOSTELIUM DISCOIDEUM: STRUCTURE, SYNTHESIS AND ROLE IN LYSOSOMAL ENZYME TARGETING.** John Bush[1], James Cardelli[1], Arnold Kaplan[2], Carlos Lacoste[2] and Hudson H. Freeze[3]. [1]LSU Medical Center, Shreveport, LA, [2]St. Louis University, St. Louis, MO and [3]La Jolla Cancer Research Foundation, La Jolla, CA.

The slime mold Dictyostelium discoideum synthesizes glycoproteins with high mannose-type N-linked oligosaccharides that contain residues of Man-6-$SO_4$ and Man-6-$POCH_3$. A small cluster of Man-6-$SO_4$ residues defines an antigenic determinant called common antigen 1 (CA1) that is shared by many proteins, including lysosomal enzymes. Two mutant strains which lack CA1 (HL241 and HL243) have oligosaccharides with a reduced $SO_4$ and $PO_4$ content because they synthesize a truncated lipid-linked precursor oligosaccharide (LLO) ($Man_6GlcNAc_2$). A third strain that lacks CA1 (HL244) makes normal-sized, fully phosphorylated oligosaccharides that are unsulfated. We have examined the effects of the partial and complete loss of sulfation on the targeting of newly synthesized lysosomal enzymes to the lysosome. Compared to wild-type, the rate of arrival of the enzymes in the lysosome is 2-3x slower in HL241 and HL243, and most of this delay can be accounted for by prolonged residence time in the RER. On the other hand, the rate of transit of the enzymes through the RER, Golgi and into the lysosome is normal in HL244. Thus, sulfate residues are not required for proper targeting, but a full-sized oligosaccharide is required for transport to occur at a normal rate.

The faithful synthesis of sulfated N-linked oligosaccharides can be carried out by incubating Golgi membranes in the presence of $^{35}S$-PAPS. About 85% of the $^{35}SO_4$ incorporated into endogeneous acceptors is sensitive to N-glycanase and displays a charge profile on HPLC that is nearly identical to total cellular $^3H$-mannose-labeled anionic oligosaccharides. The kinetics of acid hydrolysis suggest that the $^{35}SO_4$ is found 6-O-linkage to the sugars. A portion of the oligosaccharides contain Man-6-P-$OCH_3$ groups but few, if any, phosphomonoesters. In addition to the sulfation of endogeneous acceptors, ruptured Golgi vesicles can also sulfate exogenous glycoprotein acceptors derived from HL244, but not those derived from wild-type.

Thus, Dictyostelium can be a useful model system for studying the synthesis and biological function of sulfate esters on these unusual N-linked oligosaccharides. (Supported by GM32485.)

18

SULFATED N-LINKED CARBOHYDRATE UNITS OF THYROGLOBULIN: OCCURRENCE OF GALACTOSE 3-SULFATE CAPPING GROUPS AND THEIR FORMATION BY A GOLGI SULFOTRANSFERASE.
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Recent studies from our laboratory have demonstrated the presence of terminal Gal(3-$SO_4$) residues in the complex-type oligosaccharides of human and calf thyroglobulin (1) as well as in this protein from the glands of several other species. These capping groups, which are novel to N-linked carbohydrate units, are in β1-4 linkage to GlcNAc and occur alternatively to sialyl and/or α-galactosyl residues. The Gal(3-$SO_4$) groups were found to be most abundant in human thyroglobulin where they occur in amounts similar to sialic acid and are distributed among most of the numerous biantennary and multiantennary complex oligosaccharides. More internally located sulfate was also observed in a Galβ1-4GlcNAc(6-$SO_4$) sequence on some multibranched carbohydrate units. In the calf and pig thyroglobulins, which in contrast to the human, contain terminal Galα1-3Galβ1-4GlcNAc sequences (2), the sulfate was predominantly situated on the GlcNAc residues.

An enzyme which catalyzes the transfer of sulfate from PAPS to C-3 of terminal Gal residues in β1-4 linkage was found to be present in thyroid Golgi membranes (3) and this localization was consistent with the late formation of Gal(3-$SO_4$) observed during in vivo assembly of thyroglobulin carbohydrate units (4). The specificity of this sulfotransferase as assessed with exogenous glycopeptide and simple saccharide acceptors indicated that it is physiologically involved in the biosynthesis of the Gal(3-$SO_4$)β1-4GlcNAc sequence. Since N-linked oligosaccharides terminating in β-linked Gal residues can serve as acceptors for sialyl- and α-galactosyl-transferases, as well as for the 3-O-sulfotransferase, it is likely that a competition exists for the attachment of capping groups with the ultimate nature of the chain termini being to a large measure determined by the relative activities of these enzymes in a given tissue.

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## 19

NOVEL SULFATED N-LINKED OLIGOSACCHARIDES IN BOVINE PULMONARY ARTERY ENDOTHELIAL CELLS AND BOVINE LUNG

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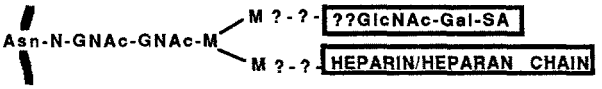
Glycosaminoglycan chains are usually attached to core proteins through a xylose residue in O-glycosidic linkage. A well known exception is Keratan Sulfate I, which is attached to the core protein via an N-linked oligosaccharide. However, KS-I can be viewed as a sulfated version of polylactosaminoglycan chains, which are commonly found on N-linked oligosaccharides. We have recently discovered a novel exception to the xylosyl-serine linkage in bovine pulmonary artery endothelial cells. Peptide:N-Glycosidase F (PNGaseF) was used to specifically release $^{35}SO_4$-labelled molecules from these cells, revealing a novel class of heparan and heparin-sulfate chains, attached to as yet unidentified proteins through an N-glycosidic linkage:




However, such molecules represented less than 10% of the $^{35}SO_4$-labelled molecules and less than 1% of the total N-linked oligosaccharides from these cells. Since lung tissue is known to be rich in endothelial cells, we searched for such structures in bovine lung acetone powder. A detergent extract was treated with PNGaseF. The released oligosaccharides were labelled by reduction with $[^3H]NaBH_4$ and fractionated by QAE-Sephadex chromatography. Of the total labelled oligosaccharides, 10-15% had properties very similar to those of the $^{35}SO_4$-labelled molecules described above. We are currently exploring their distribution in bovine lung, their interactions with molecules such as antithrombin III, and their synthesis by endothelial cells.

The same approach also showed the existence of a class of sulfated, sialylated complex-type chains in the endothelial cells. At least a portion of the sulfate esters were found in the sequence:

$$\begin{array}{l} SO_3O \\ \quad\quad | 6 \\ \text{SA } \alpha 2\text{-->(3)6 Gal } \beta 1\text{-->4 GlcNAc } \beta 1\text{-->} \end{array}$$

Similar sialylated, sulfated molecules were also found among the PNGase-F released oligosaccharides from the bovine lung extract. Further structural studies of these chains are under way.

## 20

STRUCTURE OF THE HUMAN H α(1,2)FUCOSYLTRANSFERASE GENE. John B. Lowe, Robert D. Larsen, Linda K. Ernst, and V. P. Rajan. Howard Hughes Medical Institute and Department of Pathology, Univ. of Michigan Medical School, Ann Arbor, MI 48109-0650

To begin to define the molecular basis for the regulation of expression of the blood group H gene, we have cloned, sequenced, and expressed genomic and cDNA sequences that determine expression of an α(1,2)fucosyltransferase. Chromosomal localization studies and enzyme kinetic analyses indicate that these sequences represent the human H blood group gene. Transfected mouse fibroblasts expressing this gene contain multiple transcripts homologous to cDNA sequences encoding the H α(1,2)fucosyltransferase. cDNA cloning and expression experiments indicate that each of these transcripts encodes a functional α(1,2) fucosyltransferase. Sequence analysis of these cDNAs and of the cognate gene indicate that these transcripts differ at their 5' ends. These differences can be accounted for by differential use of transcription initiation sites, and by an alternative splicing pattern that generates transcripts containing an unspliced intron. Sequence analysis indicates that the shortest transcripts have the potential to encode transmembrane proteins with short, non-cleavable signal-anchor segments at their NH2-termini, and large, catalytic domains at their COOH-termini. Preliminary sequence analysis of the longest transcript suggests that it encodes a protein consisting of 219 amino acids at the amino terminus that are encoded largely by sequences within a retained intron, followed by 352 amino acids that are colinear with the protein sequence encoded by the shorter transcripts. These observations suggest that complex regulatory mechanisms may be used to generate multiple polypeptide products from a single fucosyltransferase gene. Studies are in progress to identify these different polypeptide products in transfected cells, and to define their enzymatic and biosynthetic characteristics.

21

BASIS FOR β-GALACTOSIDE α2,6-SIALYLTRANSFERASE LOCALIZATION TO THE GOLGI APPARATUS. Karen J. Colley, Eryn Ujita Lee, *Beverly Adler, *Jeffrey K. Browne, and James C. Paulson. Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, CA 90024-1737 and *AMGen, 1900 Oak Terrace Lane, Thousand Oaks, CA 91320.

In order to investigate the basis for the retention of the β-galactoside α2,6 sialyltransferase (ST) within the Golgi apparatus, we have expressed and localized both wild type and mutant forms of the ST in Chinese hamster ovary and Cos-1 cells. The presence of soluble glycosyltransferases in body fluids suggests that some proportion of glycosyltransferases are released from their membrane anchors by proteolysis. In the case of the ST, proteolysis also occurs during the purification of the enzyme from rat liver and results in a 41 kDa form which begins with amino acid 63 of the intact, 47 kDa, Golgi form of the enzyme. These data indicated that some or all of the first 62 amino acids of the ST, which include a 9 amino acid, $NH_2$-terminal cytoplasmic tail, a 17 amino acid signal-anchor domain and an extended stem region, may be responsible for the retention of the sialyltransferase in the Golgi. To directly test this, we replaced the first 57 amino acids of the ST with the cleavable signal peptide of human gamma interferon and expressed this signal peptide-sialyltransferase (sp-ST) fusion protein and wild type ST in CHO cells. While the wild type ST was retained within the cells, the signal peptide was cleaved from the sp-ST fusion protein, resulting in the secretion of a soluble, catalytically active enzyme ($t_{1/2}$=2-3 hours). In addition, the wild type ST cDNA was altered by oligonucleotide-directed mutagenesis to contain a signal peptide cleavage site at the COOH-terminal end of the signal anchor domain. Transient expression of this signal cleavage-ST (sc-ST) in Cos-1 cells demonstrated that this altered enzyme was secreted very slowly from the cells ($t_{1/2}$ >24 hours) and the sc-ST enzyme remaining within the cells was localized to the Golgi apparatus by immunofluorscence. Another mutant, Δtail-ST, which is lacking most of the cytoplasmic tail, behaves like the wild type ST and is localized to the Golgi apparatus in the Cos-1 cells. These preliminary results suggest that the $NH_2$-terminal stem regions contains the "signal" required for ST retention within the Golgi apparatus. (Supported by NIH grants GM 27904 and GM 11557).

22

GENETIC ALTERATION OF HEPARIN BIOSYNTHESIS AND MURINE MASTOCYTOMA CELLS. Rebecca I. Montgomery, Kerstin Lindholt*, Ulf Lindahl*, and Jeffrey D. Esko, Department of Biochemistry, University of Alabama at Birmingham, Birmingham, Al 35294 and the *Department of Veterinary Medical Medicine, Swedish University of Agricultural Sciences, Biomedical Center, Uppsala, Sweden.

In order to study the function of secretory granule heparin, clonal sublines from the Furth murine mastocytoma were isolated. These sublines do not adhere appreciably to plastic substrata and divide every 12 hours in both static and stirred cultures. They produce about 1-5 μg of mixed glycosaminoglycans per $10^6$ cells (carbazole reactive uronic acid), consisting of about 85% heparin/heparan sulfate and 15% chondroitin sulfate. Nitrous acid-catalyzed deaminative cleavage of $^3$H-glucosamine-labeled heparin/heparan sulfate indicated that the chains were as heavily N-sulfated as commercial heparin. About 80% of the chondroitin sulfate contained two sulfate residues per disaccharide. Both glycosaminoglycans were initially synthesized on a core protein, subsequently liberated as free chains, and stored within cytoplasmic granules along with histamine and proteases. Treatment of cells with the calcium ionophore A23187 or with anti-DNP IgE plus DNP-hapten released about 25% of the stored glycosaminoglycan. Interestingly, little of the heparin/heparan sulfate had high affinity for antithrombin III, but treatment of the cells with sodium butyrate or starvation for inorganic sulfate induced high affinity heparin. Several mastocytoma sublines were isolated that adhere strongly to plastic tissue culture dishes and form adherent colonies efficiently. When sandwiched between the bottom of a tissue culture plate and an overlying disk of polyester cloth, the colonies transferred to the disk. Transferred colonies incorporated $^{35}SO_4$ into heparin efficiently, and the extent of incorporation was readily detected by exposure of the disk to X-ray film. Mutants defective in $^{35}SO_4$ incorporation into heparin have been isolated and their characterization is currently underway. Our ability to nutritionally and genetically modify heparin structure should allow further studies on the function of heparin in mast cell physiology.

**23**

INTERACTIONS OF SMALL PROTEOGLYCANS WITH EXTRACELLULAR MATRIX COMPONENTS AND ENDOCYTOSIS RECEPTORS. H. Kresse, H. Hausser, G. Schmidt, M. Winnemöller and W. Schlumberger. Institutes of Physiological Chemistry and Pathobiochemistry and of Arteriosclerosis Research, University of Münster, D-4400 Münster, FRG

Small dermatan sulfate proteoglycan from fibroblast secretions (PG II, decorin) is internalized by receptor-mediated endocytosis. Endosomal glycoproteins of 51 kD and 26 kD are able to bind the deglycosylated PG II core protein. The proteins coprecipitate upon incubation of endosomes with core protein and core protein-specific antibodies. Reductive methylation of lysine residues of the core protein reduces binding to a similar extent as endocytosis. Only trace amounts of 51 kD and 26 kD proteins are expressed by keratinocytes which neither produce nor endocytose PG II. These proteins are therefore proposed to be constituents of a PG II endocytosis receptor, the relation between them, however, is not yet known.

Binding of PG II to type I collagen and fibronectin interferes with endocytosis. In collagen gels the turnover of PG II is unmeasurably low. Under these culture conditions the proteoglycan is found exclusively in association with collagen fibrils. In case of the proteoglycan fibronectin interaction PG II core protein can bind to the heparin-binding domains and also to the cell-binding domain of fibronectin. Binding to the latter domain is responsible for the core protein-mediated inhibition of fibroblast adhesion to fibronectin-coated surfaces. Micromolar concentrations of the pentapeptide NKISK the sequence of which is found in several repeats of the core protein, inhibit the interaction of core protein and intact fibronectin in an ELISA test system. Binding, however, is not abolished at high concentrations of NKISK which may indicate that other core protein domains participate in the interaction with fibronectin.

**24**

THE INTERACTION OF SMALL PROTEOGLYCAN WITH COLLAGEN IN TENDON. K.G. Vogel and T.J. Koob, Dept. of Biology, The University of New Mexico, Albuquerque, NM 87131

All fibrous connective tissues that have been examined have proteoglycan constituents associated with the collagen fibrils. We have studied the interaction of proteoglycans of bovine tendon with collagen experimentally by recombination of purified components and by chemical and enzymatic extraction of tendon tissue. (1) The small proteoglycan of bovine tendon (a DSPG-II/decorin-like molecule) inhibits the rate of collagen fibril formation in *in vitro* fibrillogenesis. In contrast, large proteoglycans, glycosaminoglycan chains, and PG-I did not affect fibril formation. We have now demonstrated that both intact PG-II and its core protein generated by chondroitinase ABC bind to collagen during fibril formation ($Ka = 3 \times 10^7\ M^{-1}$), whereas neither large proteoglycans nor glycosaminoglycan chains was bound under identical conditions. When both PG-I and PG-II were added to soluble collagen and fibrils allowed to form, only PG-II was recovered from the insoluble collagen pellet. (2) The PGII core protein was not extracted from bovine tendon by incubation in NaCl concentrations ranging from 0.1 to 4M; nor was it dislodged by acetic acid at pH >4. It was released, however, by guanidine HCl above 0.4 M and by acetic acid at pH <4 - i.e., by conditions that disrupt the integrity of collagen fibrils. The proteoglycan was not removed by acetic acid (pH 2.5) when swelling of the collagen fibrils was prevented by inclusion of 1 M NaCl or 0.8 M GnHCl. (3) V8 protease preferentially clips the PG-II core protein at residue 17, liberating the $NH_2$-terminus containing the GAG and a major 40 KDa fragment. When tendon is treated with this enzyme, GAG is released from the tissue and the 40 KDa fragment remains tightly associated with the tissue, where it is resistant to further degradation. These studies indicate that: (1) the small PG-II proteoglycan of bovine tendon shows a specific, high-affinity binding to type I collagen which is mediated by the core protein; (2) this proteoglycan is associated with collagen fibrils in tendon tissue; and (3) this association with collagen fibrils *in situ* is through the core protein. The structural/mechanical role of small proteoglycans in tendon biology remains unclear. (NIH AR36110)

**25**

IMMUNOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF RAT CHONDROSARCOMA PROTEOGLYCAN. A. Calabro, M. Campbell and V. C. Hascall. Bone Research Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892.
Proteoglycan monomer was purified from the Swarm rat chondrosarcoma by associative extraction followed by sequential CsCl isopycnic centrifugation in associative and dissociative gradients (1). Clonal hybridomas were developed which produce monoclonal antibodies against epitopes within the core protein structure of the proteoglycan (2). The hybridomas were used to synthesize metabolically labeled antibodies to develop a direct binding immunodetection assay. Confluent cultures in 100 mm dishes (~15 X $10^6$ cells/dish) were equilibrated with a leucine-free medium and labeled for 24 h in the same medium with 100 $\mu$Ci [$^3$H]leucine/ml. Typically, ~70% of the incorporated radioactivity (~100 X $10^6$ dpm/dish) was present in the monoclonal antibody fraction. Proteoglycan or proteoglycan fragments were prepared in Tris/NaCl solvent and blotted at different concentrations onto polyvinylidene difluoride membranes. After blocking, the membranes were incubated with appropriate dilutions of the labeled antibody solutions followed by several washes. Membrane dots were then isolated, extracted with 4 M guanidine HCl and counted for radioactivity. For monoclonal antibodies that recognize epitopes which appear only once or twice in the core protein, such as 12-21/1C6 which recognizes a sequence epitope present twice in the protein (3), the assay is effective in the $\mu$g range. Those which recognize epitopes that are much more abundant in the core, such as 5-29/2B6 which recognizes chondroitin-4-sulfate stubs following chondroitinase digestion (4), are up to 50 fold more sensitive. Specific proteases, such as trypsin, V-8 protease and clostripain, as well as cyanogen bromide degradation are being used to generate specific mapping procedures. SDS-PAGE and western blotting will be used in combination with the labeled antibodies to determine the localization of additional epitopes within the core structure. 1. Faltz, L.L., Reddi, A.H., Hascall, G.K., Martin, D., Pita, J.C. and Hascall, V.C. (1979) J. Biol. Chem. **254**, 1375. 2. Calabro Jr., A. (1987) Ph.D. Thesis Dissertation, West Virginia University. 3. Hejna, M. (1988) Ph.D. Thesis Dissertation, Rush-Presbyterian St. Lukes Medical Center. 4. Caterson, B., Calabro, T. and Hampton, A. (1986) In "Biology of the Extracellular Matrix" (T. Wight and R. Mecham, eds.), 1-26.

**26**

REGULATION OF HYALURONAN SYNTHESIS AND DEPOSITION DURING FSH-INDUCED EXPANSION OF THE MOUSE CUMULUS CELL-OOCYTE COMPLEX. A. Salustri, M. Yanagishita and V. C. Hascall. Bon Research Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20892

In most mammalian ovaries, the cumulus cell-oocyte complex (COC) expands at the time of ovulation by depositing an extensive matrix between the cumulus cells. This expansion can be reproduced in vitro by culturing isolated COCs with follicle-stimulating hormone (FSH) and serum. Metabolic precursors, [$^3$H]glucosamine and [$^{35}$S]sulfate, were used to study the synthesis and deposition of hyaluronan (HA) and proteoglycans (PGs) in the COC matrix after stimulation with FSH. Specific activities of the [$^3$H]hexosamines in labeled molecules were determined from the $^3$H and $^{35}$S incorporation into chondroitin sulfate disaccharides in PGs. HA synthesis increased 20-30 fold between 3 and 12 hours after FSH addition to COCs, the time during which COC expansion occurs. When serum is present, the HA is deposited in the COC matrix while in its absence HA is secreted into the medium and COC expansion does not occur. The maximal rate of HA synthesis is ~780 pmol (as glucosamine)/COC/h (~1400 cumulus cells/COC), and its concentration in the expanded COC was calculated to be ~250 $\mu$g/ml. PG synthesis, while stimulated ~3 fold by FSH treatment throughout a 16 hour culture period, is at a much lower level than HA synthesis and does not correlate with COC expansion. Cumulus cells cultured with FSH and serum, but without oocytes, synthesize HA at only ~4% the level of fully stimulated COCs. In contrast, cumulus cells co-cultured with isolated oocytes or cultured with medium conditioned by oocytes for 5 h synthesize much higher levels of HA, ~50% of fully stimulated, intact COCs. PG synthesis by isolated cumulus cells is stimulated to nearly the same extent by FSH as for intact COCs even in the absence of oocytes or conditioned medium from oocytes. The data indicate that a factor(s) produced by oocytes is specifically required in addition to FSH to stimulate cumulus cells to synthesize HA, but not PGs, and that a factor(s) in serum is required to retain the newly synthesized HA in the COC matrix.

27

$^{1}$H NMR STUDIES OF THE SOLUTION CONFORMATION OF HYALURONIC ACID.

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In hyaline cartilage, proteoglycans occur as large aggregates formed when proteoglycan monomers bind to a single molecule of hyaluronate. Previous work in other labs has demonstrated that the degree and stability of aggregation depend on numerous factors, including the length of hyaluronate, pH, and ionic strength. We are using a variety of one-dimensional and two-dimensional nuclear magnetic resonance methods to study how these factors affect the conformation of hyaluronic acid fragments (human umbilical cord) and its substituent monosaccharides, glucuronic acid and N-acetylglucosamine. Preliminary results from nuclear Overhauser enhancement spectroscopy (NOESY) of short hyaluronate fragments in aqueous solutions are not consistent with previously proposed structures of hyaluronate (in non-aqueous environments). To observe exchangeable amide and hydroxyl protons in hyaluronic acid and its subunits, we are using solvent suppression pulse sequences in aqueous solutions.

28

CHARACTERIZATION OF A HYALURONAN BINDING PROTEIN;.

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A hyaluronan binding protein (HABP 68 kd), originally isolated from spent culture medium, occurs on the cell surface as a 1 x $10^{6}$ dalton complex containing the HABP and a protein kinase related to src pp60. The complex appears to be integrated in the cell membrane since it is extracted by detergents but not by high salt or urea. Expression of this HABP-complex on cell surfaces is density dependent and positively correlated with high rates of cell motility. Consistent with a role in cell locomotion, it is concentrated in the processes of rapidly locomoting cells and both polyclonal and a monoclonal antibody to HABP inhibit cell motility.

Recently a cDNA (2.8 kb) has been isolated from a γGTII 3T3 expression library by screening colonies with polyclonal and monoclonal antibodies to HABP. The nucleotide sequence of this cDNA indicates that it codes for a unique protein. Northern blot analysis indicates that the gene product of this clone is expressed as a 3.4 and 1.9 kb mRNA. Immuno-crossreactivity experiments also indicate that it is related or identical to HABP. Thus, in a transblot assay, polyclonal antibodies against HABP react with a protein from lysates of a vector containing a portion of the clone. Conversely, polyclonal antibodies prepared against a 21 amino acid sequence predicted from the 2.8 kb clone immunoprecipitate both supernatant HABP and the complex from metabolically labeled 10T1/2 cells. This polyclonal antibody inhibits both cell motility and the binding of $I^{125}$-hyaluronan to 10T1/2 cell surfaces. The distribution of the gene product detected with the polyclonal antibody to the 21 amino acid sequence of the clone is identical to that of HABP on locomoting cells using a double immunofluorescence technique. The structure of the clone and its relationship to HABP-complex is discussed.

29

POLYRIBOSOMAL ORGANIZATION IS A CONTROL POINT FOR THE RATE OF PRODUCTION OF TYPE I PROCOLLAGEN. Arthur Veis and Przemyslaw Tylzanowski. Northwestern University, Dept. of Molecular Biology, 303 E. Chicago Ave., Chicago, IL, 60611.

The pro α1 and pro α2 chains of the type I collagen heterotrimer appear to be translated coordinately in large polysome aggregates. Electron microscopic studies of the aggregates indicated that the density of loading of ribosomes on the mRNA strands was less than expected. These observations led us to consider that the production of the pro α chains in the heterotrimer might be less than if the individual mRNAs were translated independently. To examine this question the relative ribosomal loading of pro α1(I) mRNA was compared in wild type [heterotrimer producing] K16 cells and chemically transformed W8 cells [producing only pro α1(I)]. The polysomes from each cell type were isolated and fractionated on high resolution sucrose density gradients. The mRNA was isolated from each polysome fraction. Each polysome fraction was quantitatively probed for pro α1(I) mRNA on slot blots with plR1 (a generous gift from Dr. David Rowe). The pro α1(I) mRNA in the heterotrimer producing K16 cells was localized to polysomes of 15 - 20 ribosomes. The W8 cells, which produced the pro α1(I) homotrimer, had the pro α1(I) mRNA in much heavier polysome fractions, suggesting that there were more ribosomes per mRNA strand. These data support the electron microscopic observations of a low density of ribosomal loading of the mRNA in normal cells. Thus each fully loaded mRNA could be engaged in the production of more pro α chains in the transformed cells. In normal, wild-type cells, collagen production may therefore be modulated by the extent of ribosomal loading in the aggregates as well as by the amounts of mRNA and by processing related synthesis pauses. Supported by NIH Grant AR-13921.

30

EXPRESSION STUDIES WITH FULL-LENGTH cDNA CLONES WHICH ENCODE HUMAN PROCOLLAGEN CHAINS

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We have cloned full-length cDNAs encoding the human pro-alpha 2(I) and pro-alpha 2(V) collagen chains. These cDNAs have been placed downstream of strong viral promoters for expression studies in cell culture systems. Most recently, the pro-alpha 2(V) cDNA under control of the human cytomegalovirus immediate early promoter/regulatory sequences was introduced into the HT1 clonal line of Chinese hamster lung cells. These cells have previously been shown to synthesize large quantities of pro-alpha 1(V) homotrimers as their only collagenous product. Transfection resulted in a number of clonal cell lines which express human alpha 2(V) RNA at levels comparable to, and in some cases greater than, levels found in normal human fibroblasts. At the protein level, human pro-alpha 2(V) chains were produced, in the majority of clonal lines, at sufficient levels to complex all endogenous pro-alpha 1(V) chains. Chimeric heterotrimers, composed of hamster pro-alpha 1(V) and human pro-alpha 2(V) chains in a 2:1 ratio, were stable to pepsin digestion and were efficiently incorporated into extracellular matrix associated with the cell layer. Surprisingly, excess pro-alpha 2(V) chains, uncomplexed to pro-alpha 1(V) chains, were found, to some degree in matrix and in much greater abundance, in media. These chains were pepsin-sensitive, indicating that pro-alpha 2(V) chains can be secreted in a non-triple helical form. Site-specific mutagenesis of full-length cDNAs and subsequent expression in appropriate systems should provide further insight into the function and biosynthesis of various collagen chains.

## 31

THE ELASTIN RECEPTOR IS A β–GALACTOSIDE LECTIN. L. E. Grosso, and R. P. Mecham.

A high affinity elastin receptor complex has been identified on elastin producing cells. This receptor mediates a chemotactic response to elastin peptides and is involved in the organization of elastin into the extracellular matrix. A $k_d$ of 2 x $10^{-8}$M was obtained from Scatchard analysis of bovine ligamentum fibroblasts. A necessary component of the elastin binding site has been identified as the hexapeptide VGVAPG. Elastin peptide affinity chromatography has been used to isolate elastin binding proteins. Guanidine, VGVAPG, and galactoside sugars release proteins with molecular weights of 67, 61 and 55 kDaltons. These proteins remain bound to the column when non-galactose sugars or RGD containing peptides are used. Radiolabelling and subcellular fractionation studies indicate that the three proteins are located on the cell surface. Detergents are required to isolate the 61 and 55 kDa proteins and these proteins are therefore felt to be integral membrane proteins. In contrast, the 67 kDalton protein can be isolated from cells by exposure to lactose suggesting that it is a peripheral membrane protein. The ability of lactose to modulate the affinity of the 67 kDalton protein for both elastin and the other protein components of the elastin receptor indicates that this protein is a carbohydrate binding protein. The crossreactivity of the 67 kDalton protein with antibodies raised to the rat lung 14.5 kDalton β-galactoside lectin and the inhibition elastin receptor function by the addition of lactose to cell cultures are consistent with this hypothesis.

## 32

SYNTHETIC PEPTIDES OF MATRIX MOLECULES WHICH PROMOTE CELL ADHESION. Amy P. N. Skubitz, Qi Zhao, James B. McCarthy, and Leo T. Furcht. Department of Laboratory Medicine and Pathology, Box 609 UMHC, 420 Delaware Street SE, University of Minnesota Medical School, Minneapolis, MN 55455

Laminin, an 850 kD basement membrane glycoprotein, regulates the adhesion, spreading, migration, and phenotypic expression of various normal and transformed cell types. Interaction of laminin with glycosaminoglycans and proteoglycans is important in the formation of basement membranes, the adhesion of cells, and possibly the outgrowth of neurites. The amino acid sequences of the B1, B2, and A chains of laminin have been determined from the nucleotide sequence of cDNA clones. Recently, two synthetic peptides from the B1 chain of laminin termed peptide F-9 (RYVVLPRPVCFEKGMNYTVR) and peptide CDPGYIGSR have been reported to promote cell adhesion. In addition, peptide F-9 has been shown to have heparin-binding activity. In this study, these two peptides were compared for their ability to promote the adhesion and migration of the metastatic murine fibrosarcoma cell line, UV-2237-MM. Cells adhered and moderately spread in a concentration-dependent fashion on surfaces coated with peptide F-9 to about the same extent as on surfaces coated with laminin. In contrast, surfaces coated with peptide CDPGYIGSR failed to promote fibrosarcoma cell adhesion or spreading. Cells did not migrate towards either peptide F-9 or CDPGYIGSR in a chemotactic or haptotactic manner in Boyden microchemotaxis chambers in the concentration range of 1-100 μg/ml. However, exogenous soluble peptide F-9 inhibited both laminin-mediated cell adhesion and migration, while peptide CDPGYIGSR did not. Polyclonal antibodies raised against these two peptides were also contrasted for the ability to inhibit the laminin-mediated fibrosarcoma cell adhesion and migration. Only antibodies raised against peptide F-9 or intact laminin were capable of inhibiting laminin-mediated cell adhesion and migration. These results indicate that peptide F-9 represents a major cell adhesion promoting domain on intact laminin. A series of overlapping peptides were then synthesized which contained various portions of the parent peptide F-9. The amino two-thirds of peptide F-9 was as active as intact peptide F-9 at promoting cell adhesion or inhibiting laminin-mediated cell adhesion, while the amino one-third of peptide F-9 had about half of the activity. Therefore, the sequence RYVVLPR was found to be essential for cell adhesion promoting activity. These findings suggest that fibrosarcoma cell surface-associated heparin-like molecules may act as receptors or binding components for this domain of lamimin encompassed within peptide F-9.

# POSTERS

## Carbohydrate Binding Proteins

## 101

BUTYRATE INDUCES THE EXPRESSION OF A 14.5 kDa GALACTOSIDE-BINDING LECTIN IN HUMAN COLON CARCINOMA CELLS. R. Lotan, D. Carralero, D. Lotan and D. Ohannesian, Department of Tumor Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Carbohydrate-binding proteins, especially those that bind galactosides, are found in various normal and malignant tissues and cells. Galactoside-binding lectins have been localized in the cell nucleus, cytoplasm, cell surface, and in a secreted form. The levels of these lectins are regulated in certain tissues during embryonal development and they have been implicated in mediation of intercellular recognition and in cell adhesion to the extracellular matrix. The majority of tissues contain a 14.5 kDa lectin and some tissues and malignant cells contain also lectins of higher *Mr* (e.g., 29, 31, 34, and 67 kDa). During a survey of lectin expression in different tumor cells we found that several human colon carcinoma cell lines contain a 31 kDa galactoside-binding lectin but do not contain a 14.5 kDa lectin. This observation has been made by immunoblotting cell extracts using antibodies recognizing both the 14.5 kDa lectin and the 31 kDa lectin, as well as by analysis of galactoside-binding material purified from colon carcinoma cell extracts using an immobilized asialofetuin column followed by polyacrylamide gel electrophoresis and fluorography. In previous studies we have found that induction of differentiation of several tumor cell types resulted in alterations in lectin expression. In the present study the effects on lectin expression in human colon carcinoma cells of sodium butyrate, an established differentiation inducing agent for such cells, were analyzed. Several sublines derived from a human colon carcinoma surgical specimen implanted into nude mice and selected for liver colonization after intrasplenic injection (Morikawa et al., Cancer Res. 48: 1943-1948, 1988) were treated with butyrate in culture. Treatment with 2 mM butyrate caused cell flattening in some of the sublines and cell rounding in others. The treatment inhibited cell proliferation in monolayer cultures by less than 50% after 5 days, however, such a treatment resulted in a marked suppression (>90%) of the ability of the cells to form colonies in agarose. This treatment also resulted in an increase in the level of the 31 kDa lectin and, more interestingly, in the appearance of a 14.5 kDa galactoside-binding lectin, which was detected by immunoblotting as well as by purification by affinity chromatography. The induction was dependent linearly on the butyrate dose in the range between 1 and 4 mM and was detected after a 24-h treatment with 2 mM butyrate. Thus, butyrate induction of the 14.5 kDa lectin preceded any changes in cell growth. A significant 14.5 kDa lectin level persisted for several days after withdrawal of butyrate from the growth medium of the carcinoma cells. We are currently investigating the effects of butyrate on adhesive properties of the carcinoma cells.

## 102

CHARACTERIZATION OF AF/R1 PILI FROM THE RABBIT DIARRHEAGENIC *E. COLI* RDEC-1. F.J. Cassels[1], J.C. Byrd[2], P. Rafiee[2], H. Leffler[3], Y.S. Kim[2], and E. C. Boedeker[1]. [1]Department of Gastroenterology, Walter Reed Army Institute of Research, Washington, DC 20307, [2]GI Research Laboratory, VA Medical Center, San Fransisco, CA 94121, and [3]Department of Psychiatry, UCSF, San Francisco, CA 94123

*Escherichia coli* strain RDEC-1 is an enteroadherent, diarrheagenic pathogen that colonizes rabbit intestine in a species-specific manner. Although binding is mannose-resistant, the specificity for other carbohydrate moieties is unknown. AF/R1 pili, which mediate attachment of RDEC-1 to gut epithelial cells, have not been extensively characterized. AF/R1 pili were obtained from RDEC-1 cells by shearing in a Waring blender and were purified after ammonium sulfate precipitation and gel filtration on a Sepharose CL4B column. The pilus subunits were purified after depolymerization of the intact pili in saturated guanidinium chloride and gel filtration on a Superose 12 FPLC column in 8.0 M guanidinium chloride. TEM examination of the AF/R1 pili demonstrated the pili to be thin flexible rods presenting a helical appearance at high magnification. In SDS-PAGE under reducing conditions the pilus subunit ran as a single polypeptide of 19 kD. The isoelectric point of the pilus subunit in 4% polyacrylamide gels was determined to be 4.3. Amino acid analysis of the purified subunit demonstrated a large number of Asx, Ala, Thr and Gly residues with hydrophobic residues predominant. The sequence of thirty amino acid residues in the N-terminus of AF/R1 was determined. Four of the thirty residues in the N-terminus were acidic, with a single basic residue present. The AF/R1 N-terminal sequence follows a pattern described by Klemm et al., (Infect. Immun., 40, 91), consisting of alternating conserved and variant residues consistant with a predicted β-sheet, and seen in the pilus N-termini of additional *E. coli* and other bacterial strains. Both purified AF/R1 pili and antisera prepared against purified pili blocked attachment of radiolabeled RDEC-1 to immobilized rabbit intestinal brush borders. The carbohydrate specificity was indicated by the lack of binding to periodate treated brush borders. Attachment appears to involve sialic acid since sialidase treatment of brush borders and free NeuAc both decreased binding. Futher characterization of the interaction between purified pili and host cell receptors could provide a basis for interfering with attachment of diarrheagenic bacteria.

**103**

SYNTHESIS AND BIOLOGICAL ACTIVITY OF MANNOSE-6-PHOSPHATE-CONTAINING GLYCOPEPTIDE. Y. Ichikawa, H. Tomoda, Y. Ohsumi, and Y.C. Lee. Biology Department, THE JOHNS HOPKINS UNIVERSITY, BALTIMORE, MD 21218

Mannose-6-phosphate is known to be a recognition marker for targeting of newly biosynthesized lysosomal enzymes[1]. Mannose-6-phosphate receptors are located on the cell surface and intracellular compartments. In order to study binding and other properties of this receptor system and to prove a possibility of the cluster effect[2], we synthesized a radioiodinatable ligand having two terminal mannose-6-phosphate residues[3]. The structure is shown below:

$$AcNHTyrAsp[Ala\text{-}CONH\text{-}(CH_2)_5\text{-}CH_2O\text{-}Man\text{-}6\text{-}O\text{-}P]_2$$

The peptide, composed of N-acetyl-tyrosine, aspartic acid, and two alanines, was coupled with 6-aminohexyl 6-O-bis(2,2,2-trichloroethyl)phosphoryl- α-D-mannopyranoside followed by deprotection with zinc in aqueous pyridine to give the glycopeptide having two mannose-6-phosphates. Another glycopeptide having two mannose-4,6-cyclic phosphates was also synthesized from 6-O-diphenylphosphoryl mannoside derivative by treatment with triethylamine.

Inhibitory potency of the synthetic divalent ligand ($I_{50}$ 16 mM) to the binding of $^{125}$I-PMP(pentamannosylphosphate)$_{17}$-BSA to rabbit alveolar macrophages was found to be similar to that of mannose-6-phosphate ($I_{50}$ 13 mM), indicating that no cluster effect as seen in hepatic receptors[2] was observed.

1. K. von Figura and A. Hasilik, Ann. Rev. Biochem., 55 (1986) 167-193.
2. Y.C. Lee and R.T. Lee, "The Glycoconjugates," Vol. 4 (1982) 57-84.
3. Y. Ichikawa and Y.C. Lee, submitted to Carbohydr. Res.

**104**

BINDING CHARACTERISTICS OF GALAPTIN FROM HUMAN SPLEEN. R.T. LEE, Y. Ichikawa, H.J. Allen, and Y.C. Lee. Department of Biology, THE JOHNS HOPKINS UNIVERSITY, Baltimore, MD 21218

A binding assay for galaptins (β-galactoside-binding proteins, lactose lectins) was developed. In this assay, Sepharose beads to which lactose was attached via aminohexyl linkage (LacAH-Seph) was used to bind $^{125}$I-galaptin. Bound $^{125}$I-galaptin was separated from unbound by centrifuging the reaction mixture through an oil layer. Nonspecifically bound (or trapped) counts were determined using an equivalent amount of the Sepharose beads to which aminohexyl mannoside had been attached. Non-specific binding was generally less than 5%.

An inhibition assay was used to examine the specificity of a galaptin obtained from human spleen. Concentration of the inhibitor which caused 50% reduction in the $^{125}$I-galaptin binding was determined for each inhibitor. We found that: 1. Gal and GalNAc were the only monosaccharides recognized by galaptin, and the binding of GalNAc was ca. 6-fold poorer than Gal. 2. The most potent disaccharide inhibitor was LacNAc [Galβ(1,4)GlcNAc], which was 3.6-fold better inhibitor than Lac, which was in turn 146-fold better than Gal. 3. 3-Deoxy-LacNAc had 50-fold lower affinity than LacNAc. 4. It is expected that galaptins have at least two binding sites per molecule. However, there was not much increase in affinity beyond what is expected on a statistical basis when inhibitors carrying 2--6 lactosyl residues were used as inhibitors, indicating that two binding sites of galaptin probably operate independent of each other. 5. All the α-galactosides were better inhibitors than corresponding β-galactosides and thiogalatosides were better inhibitors than the corresponding O-galactosides.

## 105

ISOLATION AND PARTIAL CHARACTERIZATION OF TWO GLYCOSYLATION ISOFORMS OF LOW MOLECULAR WEIGHT PHOSPHOMANNOSYL RECEPTOR FROM BOVINE TESTES. M. Li, J.J. Distler & G.W. Jourdian. Depts. of Biol. Chem. and Int. Med., Univ. Michigan, Ann Arbor, MI 48109

Low molecular weight phosphomannosyl receptor (PMR-2) from bovine testes exhibits two isoforms on SDS-PAGE: 45 kDa (PMR-2A) and 41 kDa (PMR-2B). The isoforms present in bovine testis homogenates were partially resolved by centrifugation at 800 x g. Membranes from the 800 x g supernatant contained PMR-2B, while the 800 x g pellet contained a mixture of PMR-2A and 2B. The isoforms in the 800 x g pellet were extracted with Triton X-100 and separated by affinity chromatography on agarose-$(Man)_5$-P; PMR-2A was eluted from the column with 0.5 mM Man 6-P; a higher concentration of Man 6-P was required to elute PMR-2B. Each receptor species has the N-terminal sequence: T-E-E-K-. The amino acid composition of the isoforms is nearly identical. PMR-2A contains 34% carbohydrate and PMR-2B, 28%. Treatment of each isoform with N-glycanase yielded polypeptide cores of 29 kDa. Partial proteolytic digests of each polypeptide core gave the same peptide profile on SDS-PAGE. Digestion with endo-$\beta$-galactosidase reduced the $M_r$ of PMR-2A by 2 kDa but did not alter the $M_r$ of PMR-2B. Partial digestion with N-glycanase suggested that each isoform contains 4 N-linked oligosaccharide chains. Two chains are of the high mannose and/or hybrid type as revealed by their sensitivity to endohexosaminidase H. Treatment with neuraminidases, or mild acid hydrolysis reduced the $M_r$ of PMR-2A by 1.5 kDa; PMR-2B was unaltered. Studies with immobilized lectins indicated that the N-linked complex oligosaccharides associated with PMR-2A were largely terminated with sialic acid, and those of PMR-2B with galactose. Treatment of PMR-2A with endo-$\beta$-galactosidase or neuraminidase increased the receptor affinity for ligand, suggesting that the lower affinity of PMR-2A (compared to PMR-2B) is due to polylactosamine containing oligosaccharides and sialic acid. (Supported by NIH AM 10531)

## 106

SOLID-PHASE ANALYSIS OF SALIVARY GLYCOPROTEIN-BACTERIAL INTERACTIONS. G.G. Haraszthy*, F.A. Scannapieco, E.J. Bergey, M.S. Reddy, and M.J. Levine. Dept. Oral Biology, SUNY/Buffalo, NY 14214.

Salivary glycoproteins play an important role in modulating the adherence and clearance of oral bacteria. The former takes place on solid surfaces (e.g. salivary coated enamel) while the latter occurs in solution (e.g. saliva). Accordingly, we have used an in vitro solid phase assay to study salivary/bacteria interactions. This assay involves: 1) preparation of solid phase substrate using SDS-PAGE and electrophoretic transfer of salivary glycoproteins to Immobilon membrane; 2) incubation of immobilized salivary components with bacteria; 3) detection of bound bacteria by an enzyme-linked immunoassay utilizing appropriate rabbit anti-bacteria sera. Initial studies using human submandibular-sublingual saliva (HSMSL) and parotid saliva (HPS) resulted in the selective binding of strains of *Streptococcus sanguis* and *Streptococcus mitis* to the low molecular weight salivary mucin (MG2). *Actinomyces viscosus* interacted only with proline-rich proteins (PRP) found in both salivary secretions. To further characterize bacteria/MG2 interactions, asialo-MG2 was prepared. As before, binding to native MG2 could be detected, however none of the streptococcal strains tested above bound to the asialo-derivative. This sialic acid-dependent binding could also be demonstrated using the serum glycoprotein, fetuin, and its asialo-derivative. To begin characterizing the streptococcal adhesins, bacterial surface proteins were extracted from *S. sanguis* with 2 mM sodium barbital or with 0.3 M lithium diiodosalicylate, dialyzed and lyophilized. Both extracts were tested in the solid-phase binding assay and showed binding activity to MG2. These findings suggest that the binding of oral bacteria to solid-phase salivary molecules is selective and mediated by sialic acid-containing glycoproteins in the case of *S. sanguis*. This technique may be useful for further study of bacterial adhesins. Supported by USPHS Grants DE08240 and DE07585.

**107**

CHARACTERIZATION OF MANNOSE-BINDING LECTINS FROM DAFFODIL AND AMARYLLIS BULBS. Hanae Kaku, Irwin J. Goldstein, Els J. M. Van Damme, and Willy Peumans. Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109, and Lab. voor Plantenbiochemie, W. de Croyaan 42, B-3030 Leuven, Belgium

The carbohydrate binding specificities of the daffodil (Narcissus pseudonarcissus;NPA) and amaryllis (Amaryllis belladonna) lectins, isolated by affinity chromatography on immobilized mannose, were studied by quantitative precipitation, sugar hapten inhibition and affinity chromatography on the immobilized lectins. These lectins precipitated stongly with several yeast mannans, but not with glucans (dextrans and glycogen). Both lectins also precipitated with yeast galactomannans containing galactosyl end groups, a synthetic linear α-1,6-mannan, an α-1,3-mannan (DP=30) and its periodate-oxidized, reduced derivative. These observations suggest that NPA and amaryllis lectins recognize both terminal and internal monnosyl units.

D-Mannose was the best monosaccharide inhibitor whereas D-Glc and D-GlcNAc were non inhibitors for both lectin precipitation systems. The best oligosaccharide inhibitors of NPA interaction were α-1,6-linked Man units, which were 5 to 12 times more potent than D-Man. Furthermore, Manα1,6Manα1,6Man was twice as good an inhibitor as Manα1,6Man-α-O-Me and 10 times better than Me-α-D-Man. On the other hand, α-1,3- and α-1,6-linked Man units were good inhibitors (6 to 20-fold more active than D-Man) of amaryllis lectin-mannan precipitation system.

Various asparagine-glycopeptides containing α-1,6-Man units were retarded on the immobilized NPA column; glycopeptides with α-1,3- or α-1,6-mannosyl residues showed greater retardation on the immobilized amaryllis column; $Man_5GlcNAc_2$-Asn (containing two Manα1,3(Manα1,6) units) bound to this column. Glycopeptides with hybrid type glycan chains were not retarded on either column.

Amino acid sequence studies of NPA showed 84% homology with those of the amaryllis and snowdrop lectins; the homology between amaryllis and snowdrop was 79%.

This research was supported by NIH Grant GM 29470.

**108**

AMINO ACID SEQUENCE HOMOLOGY OF PROTEINS THAT BIND SULFATED GLYCOCONJUGATES.

G.D. Holt, and V. Ginsburg; Laboratory of Structural Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA

Properdin, which stabilizes the C3 convertase during the activation of the alternate complement pathway, contains amino acid sequence homologies with several proteins that bind sulfated glycoconjugates, for example the adhesive protein thrombospondin and the leech salivary protein antistasin (see below). To determine if this homologous amino acid sequence is a sulfated glycoconjugate-binding domain, properdin was examined for binding to various lipids in solid phase radioimmunoassays. Of the lipids tested, properdin binds with high affinity only to sulfatide [Gal(3-$SO_4$)β1-1Cer], and does not bind to comparable levels of phosphatidyl serine, GM1, phosphatidyl ethanolamine, galactosyl ceramide, or cholesterol-3-$SO_4$. The binding of properdin to sulfatide is inhibited by dextran sulfate 500,000, and heparin, with $I_{50}$ values of 7.2 and 220 μg/ml respectively. Comparable levels of chondroitin sulfates A, B, C, keratan sulfate, dextran sulfate 5,000, fucoidan, or hyaluronic acid do not inhibit binding. Taken together, these data suggest that properdin, like thrombospondin and antistasin, binds sulfated glycoconjugates, and that the sequences shown below may be sulfated glycoconjugate-binding domains. Currently, malaria sporozoites are being examined for binding to sulfatide since coat proteins from this parasite contain homologies with the sequences shown below.

| Protein | Sequence | Residues |
|---|---|---|
| Properdin | C S V T C S E G S Q L R H R | 62-75 |
| Thrombospondin | C S V T C G G G V Q K R S R | 486-499 |
| Antistasin | C R V H C P H G F Q - R S R | 33-45 |

## 109

INVASION OF HEp-2 CELLS BY *SALMONELLA TYPHIMURIUM*. R.K. Ernst, D.M. Dombroski, D.W. Dyer and J.M. Merrick, Department of Microbiology, SUNY Buffalo, Buffalo, New York 14214

Members of the genus Salmonella are characterized by their ability to invade epithelial cells as an early step in their pathogenesis. Adherence is generally presumed to be a prerequisite for invasion and we have previously shown that S. typhimurium strains with type 1 fimbriae (mannose sensitive (MS) adhesins) adhered to isolated rat enterocytes in much higher numbers than did related nonfimbriated strains. Studies on the invasion of HEp-2 cells by S. typhimurium revealed that MS fimbriae promoted invasion but were not absolutely required and therefore played a role as accessory virulence factors. Other factors which influence invasion were, growth phase and anaerobiosis. Bacterial cells obtained from the logarithmic phase of growth invaded HEp-2 cells at higher levels than did stationary phase cells. Bacterial cells maintained in the stationary phase of growth for extended periods of time lost their ability to penetrate cultured epithelial cells. Anaerobiosis also promoted invasion which suggests that reduced oxygen levels may serve as an environmental signal for induction of proteins necessary for the invasion process. Mu dJ prophages were used to generate operon fusions and anaerobically sensitive mutants were isolated that have reduced capacity to invade HEp-2 cells.

## 110

CARBOHYDRATE-BINDING SPECIFICITY OF HUMAN SPLENIC GALAPTIN. A. Sharma, H.J. Allen, H. Ahmed and K. Matta. Roswell Park Memorial Institute, Buffalo, NY 14263

A galactose-binding lectin (galaptin) from human spleen has been purified to homogeneity by affinity chromatography on asialofetuin-Sepharose. The native lectin is a 30 KDa dimer composed of identical 14.5 KDa subunits. The carbohydrate-binding specificity of galaptin has been investigated by analyzing the binding of the lectin to asialofetuin in the presence of putative inhibitors. An ELISA method was developed which involved adsorption of asialofetuin to microtiter plates. Galaptin bound to asialofetuin was detected with polyclonal rabbit anti-galaptin serum followed by goat anti-rabbit IgG-peroxidase conjugate. Concentration of inhibitors giving 50% inhibition were graphically determined and normalized relative to lactose. These analyses revealed that galaptin has a combining site at least as large as a disaccharide. The disaccharides having non-reducing terminal galactosyl residues linked beta-glycosidically 1,3; 1,4; 1,6 to D-Glc or D-GlcNAc are better inhibitors than free D-Gal. 2-Ø-Methyl N-acetyllactosamine, N-acetyllactosamine, p-aminophenyl-beta-lactoside, lactose and beta-D-Gal(1-->3) alpha-D-GlcNAc 1-->ØMe are 780, 510, 130, 50 and 130 times as active as D-Gal. GalNAc, either free or glycosidically linked, appears to have no affinity for the lectin. The results obtained with methyl and nitrophenyl galactosides indicate that hydrophobic interactions may also occur. The nitrophenyl galactosides are better inhibitors than the methyl galactosides; hydrophobic interaction appears to enhance binding of the alpha-galactosides relative to the beta-galactosides. Gal beta 1-->3 GlcNAc beta 1-->3 Gal beta 1-->4 Glc is considerably more potent than Gal beta 1-->3 GlcNAc beta 1-->3 Gal beta 1-->ØMe indicating the possibility of an extended binding site. The data indicate that lactosaminoglycans and cell surface glycolipids are likely galaptin receptors in vivo.

PHYSICO-CHEMICAL CHARACTERIZATION OF HUMAN GALAPTIN. H.J. Allen, A. Sharma and R. Chemelli. Roswell Park Memorial Institute, Buffalo, NY 14263.

Soluble beta-galactoside binding lectins (galaptin) are widely distributed in vertebrate tissues and the most commonly studied is a dimeric beta-galactoside binding lectin with a subunit of $M_r$ 14.5 KDa. To elucidate the physico chemical nature of such lectins in lymphoid tissues, the present study was undertaken. We isolated one such lectin from human spleen tissue by affinity chromatography on asialofetuin-Sepharose. Molecular weight and isoelectric point of isolated lectin was estimated by SDS-PAGE and IEF-PAGE, respectively. For sequencing, enzymatically/chemically digested samples were blotted onto PVDF membrane following SDS-PAGE. Bands of interest were cut out and sequenced directly on a gas-phase sequencer. The isolated lectin showed a subunit $M_r$ 14.5 KDa and on IEF-PAGE focused as three distinct bands corresponding to pI's of 4.6, 4.80 and 4.85 respectively. The optimal pH for ligand-lectin interaction was found to be 7.5. It has a molar extinction coefficient of $8 \times 10^3$ at 280 nm, and on CD analysis showed 40% beta-sheets and only about 10% or less alpha-helix. There was no change in the conformation following ligand (lactose) binding to lectin. On sequencing, the amino terminus of the spleen lectin was found to be blocked. Out of 137 amino acids, 94 have been sequenced and show complete homology with human placental lectin sequence reported by others. A 14.5 KDa band was detected on Western blots of splenic tissues of many other mammalian species, human buffy coat cells and EBV-immortalized human B lymphoblastoid cells.

These results show that human splenic galaptin is probably identical to placental, buffy coat and B lymphoblastoid cell galaptin and that galaptin with a similar subunit is found in several mammalian spleens.

# Glycolipids

**112**

ISOLATION OF GANGLIOSIDES DISPLAYING A GALα1-3GAL EPITOPE IN PORCINE KIDNEY. Stephen P. Hendricks and Bruce A. Macher, Department of Chemistry and Biochemistry, San Francisco State University, San Francisco, CA 94132.

A minor ganglioside containing a non-reducing terminal Galα1-3Gal disaccharide has been identified in porcine kidney. Detection of the Galα1-3Gal epitope was achieved using a mouse monoclonal antibody, Gal-13, which specifically recognizes glycosphingolipids with a Galα1-3Gal terminus. Antibody binding was monitored using a TLC immunostaining technique and an enzyme-linked immunosorbant assay (ELISA). Binding of Gal-13 to the glycosphingolipid could only be detected after removal of sialic acid by neuraminidase. Treatment with α-galactosidase eliminated Gal-13 binding. This result along with TLC migration and glycosidase degradation studies suggest that the ganglioside is a branched (biantennary) compound containing the Galα1-3Gal epitope on one terminus and sialic acid on the other. A ganglioside with similiar properties was also detected in sheep, but not rat, guinea pig, rabbit or bovine kidney glycolipids. Purification and structural characterization studies are currently underway. This work was supported in part by NIH grant GM40205.

**113**

STRUCTURAL CHARACTERIZATION OF GALα1-3GAL GLYCOLIPIDS ISOLATED FROM KIDNEY OF VARIOUS NON-PRIMATE MAMMALS AND NMR ANALYSIS ON NEUTRAL GLYCOSPHINGOLIPIDS FROM PIG KIDNEY. Keyvan Jalali-Araghi and Bruce A. Macher. Department of Chemistry and Biochemistry, San Francisco State University, San Francisco, CA 94132

The epitope Galα1-3Gal has been observed at the non-reducing end of the glycoconjugates of non-primate mammals and New World monkeys, whereas humans, apes, and Old World monkeys contain this epitope in a cryptic form on red blood cells. The expression of this epitope in humans, apes, and Old World monkeys has apparently been suppressed by the presence of a relatively large amount of a naturally occurring antibody, anti-Gal, which specifically binds this epitope. To further evaluate the structure of the Galα1-3Gal glycoconjugates we analyzed kidney tissues of the following organisms: sheep, rabbit, cow, pig, rat, and Guinea pig. A mouse monoclonal antibody, Gal-13, which also specifically binds the Galα1-3Gal epitope was used to detect the immunoreactive glycolipids. These compounds were extracted from kidney tissues and purified by chromatography. Enzyme-linked immunoassays revealed the presence of this epitope in the kidney of all the animals except rat. TLC immunostaining of glycolipids from the five other animals showed an immunoreactive component that cochromatographed with a ceramide pentahexoside standard, $IV^3$-α-Gal-nLc$_4$Cer. Sheep had a more complex neutral glycolipid containing the epitope which cochromatographed with $VI^3$-α-Gal-nLc$_6$Cer. Pig contains an even longer chain neutral glycolipid with this epitope which we are currently isolating for structural analysis.

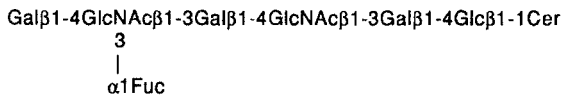
$^1$H-NMR spectrometry is being used to elucidate the structure of the neutral glycolipids isolated from kidney. Analysis of pig kidney ceramide tri- and tetra-hexoside has characterized these compounds as members of the globo series. The rest of the neutral glycolipids in the pig kidney are currently being investigated by $^1$H-NMR. Further experiments such as selective decoupling and two-dimensional NMR will be used to more completely elucidate the structure of these compounds. Confirmation of structural assignment will also be done by GC-MS analysis of permethylated alditol acetates, as well as FAB-MS. This work was supported in part by NIH Grant GM40205.

## 114

STRUCTURAL CHARACTERIZATION OF GLYCOSPINGOLIPIDS RECOGNIZED BY A MYELOID SPECIFIC MONOCLONAL ANTIBODY, VIM-1. Raymond K. Kong and Bruce A. Macher, Department of Chemistry and Biochemistry, San Francisco State University, San Francisco, CA 94132.

A mouse monoclonal antibody, VIM-1, specific for human myelogenous leukocytes, was found to bind to neutral glycospingolipids isolated from human chronic myelogenous leukemia (CML) cells. The neutral glycospingolipids were purified by various methods including DEAE-Sephadex column chromatography, Florisil column chromatography of acetylated derivatives and high performance liquid chromatography. Isolation of the immunoreactive glycospingolipids was aided by a sensitive enzyme-linked immunosorbent assay procedure used in conjunction with thin layer plate immunostaining studies.

Structural information for one of the immunoreactive glycospingolipids was obtained using mild acid hydrolysis, exo- and endo-glycosidase treatment in conjunction with immunostaining studies with carbohydrate sequence specific antibodies. These studies show that the neutral glycospingolipid recognized by VIM-1 has the following structure:




The structure of VIM-1 antigen will be confirmed by combined gas chromatography-mass spectrometry of the partially permethylated alditol acetates. Several other more complex CML neutral glycospingolipids are also immunoreactive with VIM-1. These glycospingolipids have been purified and will be structurally characterized. This work was supported in part by NIH Grant CA32826.

## 115

THE PRESENCE OF A THIRD ALLELE ON THE *Ggm-1* LOCUS WHICH REGULATES POLYMORPHIC EXPRESSION OF GM1 AND GD1a IN MOUSE LIVER. Y. Hashimoto[1], M. Sakaizumi[2], Y. Nakamura[3], K. Moriwaki[4], T. Yamakawa[3] and A. Suzuki[3]. [1]Dept. Biochem., Univ. Mass. Med. Ctr., [2]Dept. Lab. Anim. Sci. and [3]Dept. Memb. Biochem., Tokyo Metrop. Inst. Med. Sci. and [4]Natl. Inst. Genetics

In the previous paper we reported that the amounts of GM1 and GD1a ganglioside in mouse liver gradually decreased with growth or aging and that the rate of the decrease differed among inbred strains of mice. The contents of GM1 and GD1a in total gangliosides in C57BL/10 (B10), A and SWR/J mice were 1, 20 and 60% at 4 weeks and had decreased to 0, 10 and 40%, respectively, at 8 weeks. The results of a mating experiment between B10 and SWR/J mice indicated that the phenotype of SWR/J was dominantly inherited by the progeny mice and regulated by a single autosomal gene, designated as *Ggm-1*. *Ggm-1* was located 1 cM centromeric to H-2 complex, major histocompatibility complex of mice, on chromosome 17.

In the present study we performed a mating experiment between B10 and A mice and found that the phenotype of A mice was inherited by the $F_1$ hybrids and was genetically linked to a H-2 haplotype in the backcross of the $F_1$ to B10 mice. These results suggest that A mice, which show a different phenotype from both SWR/J and B10 mice, have a third allele on *Ggm-1*. Unexpectedly B10.A mice were found to have significantly higher amounts of GM1 and GD1a than both B10 and A mice, although they were a H-2 congenic strain carrying the H-2 region genes transferred from A mice and were assumed to have the same gene on *Ggm-1* as A mice. It remains to be determined how B10.A mice have different phenotype from both B10 and A mice.

**116**

EFFECT OF CHEMICAL MODIFICATION ON AMINO ACIDS IN β-HEXOSAMINIDASE A, GM2-ACTIVATOR AND GM1-ACTIVATOR. S. Nagarajan, Y.-T. Li and S.-C. Li Department of Biochemistry, Tulane University, School of Medicine, New Orleans, La. 70112

Covalent modification of amino acids by specific reagents was used to elucidate the role of amino acids in the catalytic site(s) and the binding site(s) of β-hexosaminidase A (Hex A), GM2-activator (Li, et. al., JBC 256, 6234-6240, 1981) and GM1-activator (Li, et. al., JBC 263, 6588-6591, 1988). Since the active site(s) and the binding site(s) of an enzyme can be protected from chemical modifications using substrate analogues or competitive inhibitors, protection studies were also performed.

The modifications of His, Arg, Tyr, and Cys inactivated Hex A activity toward 4-methylumbelliferyl-GlcNAc (MUG), MU-GlcNAc-6-$SO_4$ (MUGS) and GM2 to approximately the same extent, whereas, the modification of Lys and Trp affected the hydrolytic activity of MUG and MUGS more than that of GM2. The results of protection studies showed that the active site(s) of Hex A involved in hydrolyzing these three substrates may be very close to each other. Furthermore, GM2-activator did not directly affect the catalytic site(s) of Hex A, and seemed to bind to the enzyme at a separate site.

As to GM2-activator, the modification of Lys did not affect the activator activity and the modification of Arg, Cys, His, Tyr, Trp and -COOH group all showed 20 to 50% reduction of the activity. This reduction of the activity could be almost fully protected by GM2 or Hex A. Our result suggests that the expression of the GM2-activator activity probably also involves hydrophobic amino acids.

The result of modification of GM1-activator showed a different pattern as compared to that of GM2-activator. Among the six amino acids and -COOH group studied, only the modification of Trp reduced 45% activity, and that of -COOH reduced 14% of the activity. Furthermore, these inactivations were not protected by GM1 or β-galactosidase. These results indicate that the expression of GM1-activator activity does not involve the same amino acids as for that of GM2-activator. Hydrophobic interaction probably is the major mechanism of action for GM1-activator. (Supported by NIH NS 09626 and NSF DMB 8617033).

**117**

ADHERENCE OF *P. AERUGINOSA* TO NEUTRAL GLYCOSPHINGOLIPIDS OF RABBIT CORNEAL EPITHELIUM

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Departments of Ophthalmology,* Biochemistry,+ and Medicine,+ Tufts University School of Medicine, New England Medical Center,# Boston, Mass, and E.K. Shriver Center,‡ Waltham, Mass.

A thin-layer chromatogram overlay assay was used to demonstrate binding of $^{35}S$-labeled *P. aeruginosa* to neutral glycosphingolipids of rabbit corneal epithelium in culture. These cells, grown in tissue culture, underwent Folch extraction to isolate a lower phase containing neutral glycosphingolipids (NGSL) and an upper phase containing gangliosides. Using a dot blot assay, at least 6 times more radiolabeled *P. aeruginosa* were shown to bind to the lower phase compared to the upper phase. Thin-layer chromatography of the lower phase followed by staining with an orcinol spray revealed at least 10 NGSL components. Using a thin-layer chromatogram overlay assay, $^{35}S$-labeled *P. aeruginosa* was shown to bind to NGSL components 1, 2, 5, 6 and 9. Whether any of the five *P. aeruginosa*-reactive NGSL of corneal epithelium identified in this study plays a role in the development of corneal infection remains to be determined. However, of the five *P. aeruginosa*-reactive NGSL identified in this study, component #9 was present in a significantly greater amount in migrating epithelium as compared to nonmigrating epithelium and may prove to be of biologic significance because it is generally believed that traumatized/migrating epithelium is more susceptible to infection than normal/nonmigrating epithelium.

## 118

EVIDENCE THAT GANGLIOSIDE GD3 PLAYS A CENTRAL ROLE IN BLOOD PLATELET FUNCTION BY MODULATION OF THE INTEGRIN RECEPTOR GLYCOPROTEIN IIb/IIIa. T.A.W. Koerner, L.S.B. Bullard, K.E. Walker, T.T. Do, H.M. Dunniway, J.A. Barton and J.D. Olson, Dept. of Pathology, University of Iowa College of Medicine, Iowa City, IA 52242.

We have recently reported that human platelets (PLTs) contain the disialo ganglioside GD3 (NeuAcα2→8NeuAcα2→3Galβ1→4Glcβ1→1'Ceramide). This finding, together with the observation of Cherish *et al.* (JCB 105, 1163, 1987) that the binding of vitronectin to its receptor is mediated by ganglioside GD2, suggested to us that the related integrin family receptor of platelets (glycoprotein IIb/IIIa) may also require a disialo ganglioside for its function. Thus, we examined the influence of an anti-GD3 monoclonal antibody (R24) on PLT aggregation. Preincubation of $1.2X10^8$ PLTs in plasma with R24 significantly inhibited PLT aggregation induced by the agonists ADP, arachidonate, collagen, thrombin and epinephrine (secondary response). For example, the half maximal rate of aggregation in response to ADP (0.5μM) was inhibited by 400 ng/ml (2.7 nM) of R24. Based on these findings, we propose that the key first step in PLT stimulation, the PLT "shape change", is the agonist-induced association of the PLT integrin receptor (glycoprotein IIb/IIIa) with ganglioside GD3 and exposure of this complex at the PLT surface. Subsequent binding of $Ca^{++}$ and integrin ligands (fibrinogen, fibronectin, von Willebrand factor and/or vitronectin) to this activated complex would then result in PLT aggregation and adhesion. Support for this model has recently been presented by Santoro (Blood 73, 484, 1989) who has shown that platelet adhesion to immobilized fibrinogen, fibronectin and von Willebrand factor are all inhibited by brain gangliosides. Though no GD3 was used in the Santoro study, it is significant that the most effective inhibitor found was GT1b, the only ganglioside used that contains the NeuAcα2→8NeuAc fragment, which is characteristic of the disialo gangliosides GD3 and GD2. (Supported by USPHS-NIH Grant HL-42395-01)

## 119

GLYCOPHOSPHOSPHINGOLIPIDS FROM TRITRICHOMONAS FOETUS: ISOLATION AND CHARACTERIZATION. B.N. Singh[1], C.E. Costello[2], D.H. Beach[1], and G.G. Holz, Jr.[1]. [1]Microbiology & Immunology Dept., SUNY HSC, Syracuse, NY; [2]Chemistry Dept., M.S. Facility, MIT, Cambridge, MA;

In recent years, glycosylated phosphatidylinositol glycolipids have been detected as membrane anchors for a wide variety of eukaryotic cell proteins. Also there is some evidence that certain protozoans have similar glycoconjugates anchored by a ceramide moiety. We have used metabolic labeling and chromatographic and mass spectrometric techniques to characterize the glycophosphosphingolipids of Tritrichomonas foetus, an aerotolerant, anaerobic parasite of the urogenital tract of cattle. The acidic glycolipid fraction of T. foetus obtained by DEAE Sephadex A-25 column chromatography was subfractionated by HPTLC and the component lipids purified by HPLC. Two of the lipid fractions, designated $TF_1$ and $TF_2$, could be metabolically labeled with [$^3$H]myo-inositol and with [$^{32}$P]orthophosphate, but had chromatographic properties distinct from phosphatidylinositol. $TF_1$ could also be labeled with [$^3$H]fucose and partially hydrolyzed by α-fucosidase. Both lipids contained ceramides. The major ceramide contained the 18:0 and 18:1 bases and the 16:0 N-acyl group. In $TF_1$ inositol diphosphate is present, linked to fucose, (through phosphate) to the ceramide, and (through the second phosphate) to methyl, ethanolamine or N-acetylethanolamine. $TF_1$ appears to be a novel class of glycophosphosphingolipid. We are currently in the process of elucidating the complete structures of these unique compounds.

GLYCOLIPID AND GLYCOPROTEIN TRANSPORT BETWEEN GOLGI COMPARTMENTS PROCEED BY BIOCHEMICALLY INDISTINGUISHABLE MECHANISMS. THE RECONSTITUTION OF GLYCOLIPID TRANSPORT THROUGH THE GOLGI IN A CELL FREE SYSTEM. B.W. Wattenberg. Cell Biology, The Upjohn Company, Kalamazoo, MI 49001

The work of Pagano and associates (Lipsky, N.G. and Pagano, R.E. 1985. J. Cell Biol., 100, 27-34.) has indicated that glycolipids progress through the Golgi and to the plasma membrane by a vesicular mechanism. However little is known about the molecular details of this important pathway. A particularly important unanswered question is whether glycoproteins and glycolipids are co-transported. Here I report the reconstitution of glycolipid transport between compartments of the Golgi in a cell free assay. The passage of glycolipid from one compartment to the next is marked by a glycosylation event in a similar manner to that used previously to measure glycoprotein transport to the trans Golgi (Rothman, J.E. 1987. J. Biol. Chem. 262,12502-12510). Using this assay I have compared glycolipid transport to transport of the membrane glycoprotein ("G" protein) of vesicular stomatitis virus. Both have requirements for nucleotide triphosphates, elevated temperature, intact membranes and a high molecular weight cytosolic fraction. It was found that the two processes were also indistinguishable kinetically. Furthermore both glycoprotein and glycolipid transport exhibited a requirement for the recently isolated NEM Sensitive Factor. In aggregate these data suggest that glycolipid and glycoprotein transport through the Golgi share a common transport mechanism.

Immunochemistry and Immunobiology

**121**

IMMUNOLOGICAL AND HISTOCHEMICAL STUDIES ON THE PATHOPHYSIOLOGY OF GROUP B STREPTOCOCCAL TOXIN AS MANIFESTED IN EARLY ONSET DISEASE. B. Russell, R.S. Pappas, J. Brandt, H. Sundell, and C.G. Hellerqvist. Vanderbilt University School of Medicine, Department of Biochemistry, Nashville, TN 37232.

Group B streptococcus (GBS) causes "early onset" disease in newborns resulting in pulmonary edema, increased pulmonary arterial pressure and vascular permeability, fever and chills. We have previously demonstrated that an exotoxin, GBS toxin, can be isolated from the supernatant of GBS cultures obtained from infants who died of early onset disease. When infused in sheep, GBS toxin accumulates in the lung tissue, and induces the pathophysiological characteristics of "early onset disease". In this study GBS toxin, polysaccharide in nature and active at $10^{-11}$ moles per kg when infused in a lamb, was used as an immunogen to produce polyclonal rabbit anti-GBS toxin IgG. Purified anti-GBS toxin IgG was positive against GBS toxin in a microprecipitin reaction. GBS toxin when passed through a protein-A affinity column with absorbed anti-GBS toxin IgG's was shown to produce a reduced pathophysiologic response in sheep relative to toxin samples passed through protein A columns with or without absorbed preimmune IgG.

Rabbit anti-GBS toxin IgG were also used for peroxidase-anti-peroxidase localization of toxin binding sites in sheep lung. Ten micron frozen thin slices of sheep lung tissue were incubated with GBS toxin followed by immune or preimmune IgG. Photomicrographs taken at 1000 x magnification showed marked staining of capillaries and arterioles relative to control sections which were incubated with preimmune IgG, or anti-GBS toxin IgG in the absence of GBS toxin. Thus, this data suggests that a GBS toxin/receptor complex is involved in the pathogenicity of early onset disease.

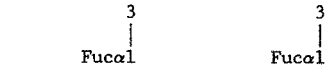
This work was supported by grants from the March of Dimes Foundation and the National Institute of Health (HL22520 and BRSG RR05424).

**122**

MONOCLONAL ANTIBODY 624H12, WHICH DETECTS LUNG CANCER AT EARLY STAGES, RECOGNIZES A SUGAR SEQUENCE IN THE GLYCOSPHINGOLIPID DIFUCOSYLNEOLACTO-NORHEXAOSYLCERAMIDE ($V^3FucIII^3FucnLc6Cer$)

M. Kyogashima, J. Mulshine, J.L. Magnani, E. Nudelman, S. Hakomori, and V. Ginsburg. National Institutes of Health, Bethesda, MD 20892, and the Biomembrane Institute and University of Washington, Seattle, WA 98119, USA.

Immunocytochemical staining of cells in sputum by rat monoclonal antibody 624H12 detects the presence of lung cancer two years prior to its detection by conventional diagnostic techniques such as sputum cytology evaluation or chest x-ray [Tockman *et al* J. Clin. Oncol. 6:1685-1693, 1988]. As shown by immunostaining of thin layer chromatograms, antibody 624H12 binds to several neutral glycosphingolipids extracted from various cell types. Studies with purified glycosphingolipids show that the antibody binds avidly to a difucosylneolactonorhexaosyl-ceramide whose structure is:




Both fucosyl residues are required for high affinity binding by the antibody. This glycosphingolipid was originally isolated from human colonic and liver adenocarcinomas, and is either absent or present in undetectable levels in normal colonic mucosa or liver [Hakomori *et al* J. Biol. Chem. 259:4672-4680, 1984].

## 123

ANTIBODIES DIRECTED AT THE CARBOHYDRATE UNITS OF GLYCOCONJUGATES, GLYCOPROTEINS AND HETEROGLYCANS. John H. Pazur, Frank J. Miskiel and Timothy F. Witham. Biochemistry Program, The Pennsylvania State University, University Park, PA 16802

Glycoconjugates, glycoproteins and heteroglycans, when used to immunize rabbits, stimulate the immune system to produce antibodies which exhibit specificity for the carbohydrate units of the immunogens. These antibodies are anti-carbohydrate antibodies and have been isolated from rabbits immunized with the glycoconjugates Gal-BSA, Glc-BSA, Man-BSA, and Fuc-BSA or with glycoproteins containing Man oligosaccharides or with heteroglycans containing Lac, GlcUA, GlcNAC or Ara*f*-(1,4)-GlcUA side chains. Some of the glycoconjugates also induce the formation of anti-BSA antibodies which are easily removed by affinity adsorbents. The anti-carbohydrate antibodies were purified by affinity chromatography on adsorbents bearing the proper carbohydrate ligands. Inhibition of the antibodies in the precipitin reactions by various carbohydrates were studied by a micro-diffusion method. In each case the antibody-antigen precipitin reaction was inhibited by the carbohydrate which constitutes the non-reducing end of the side chains of the immunogens. By isoelectrofocusing, the purified antibody preparations were found to consist of isomeric forms of the antibody ranging from 4 to 23 molecular types. The isomeric forms exhibited antibody activity as revealed by an assay method coupling isoelectrofocusing and agar diffusion. The individual members of several sets of anti-carbohydrate antibodies were isolated by preparative isoelectrofocusing. All of the pure components reacted with the antigen in the precipitin test. The anti-carbohydrate antibodies have been used to investigate the nature of light and heavy chain interactions, the identification of the origin of glucoamylases which hydrolyze starch and, by selecting cross-reacting antibodies, the detection of glycoproteins in normal and diseased cells.

## 124

ANTI-COLONIC ADENOCARCINOMA MONOCLONAL ANTIBODIES NR-CO-01,03,04 AND 05 RECOGNIZE THE $Le^a$ CARBOHYDRATE STRUCTURE. E.J. Nichols, P.K. Shadoan, D. Libby, S. Graves, A.C. Morgan, C.S. Woodhouse. NeoRx Corp. 410 W. Harrison, Seattle, WA 98119

The development of the murine monoclonal antibodies NR-Co-01,03, 04 and 05, which are reactive with human colonic adenocarcinoma while exhibiting restricted normal tissue reactivity, was previously described (C.S. Woodhouse and A.C. Morgan, Jr., 1989., Cancer Research. 49:2766-2772). The antibodies were found to react with the non-polar phase of a Folch partition and a glycoprotein extracted from ovarian cyst mucin, suggesting recognition of a carbohydrate structure(s).

We have now shown that the reactivity resides in the purified neutral glycolipid fraction obtained from the lower phase of a Folch partition of LS180 cell extract. The reactive neutral glycolipid was purified and the carbohydrate structure was shown to be identical to Lacto-n-fucopentose II by TLC. Competition inhibition ELISA using various oligosaccharide structures showed a 50% and 98% inhibition of binding of NR-Co-04 to LS180 cell extracts by LNF-II at 13 uM and 200 uM respectively. These antibodies were capable of inducing C′-mediated lysis of LS180 tumor cell targets, but not of $Le^a$ positive red blood cells which bore low levels of LNF II. Comparison with other $Le^a$-reactive antibodies by solid phase ELISA and competition inhibition ELISA demonstrates significantly lower reactivity of NR-Co-04 with the glycolipid form present in $Le^a$-positive normal human serum.

**125**

MODULATION OF GANGLIOSIDE BIOSYNTHESIS IN LEUKEMIC T-LYMPHOBLASTS BY PHORBOL DIESTER, INTERLEUKIN 2 AND INTERFERON. W.D. Merritt, C. Kueter and G.H. Reaman. Children's National Med. Ctr., Washington, DC 20010, and The George Washington University Med. Ctr., Washington, DC 20037.

The major gangliosides of leukemic cells in human T-cell acute lymphoblastic leukemia (T-ALL), $GM_3$ (hematoside) and $GD_3$ (disialolactosylceramide), are also synthesized by certain T-ALL cell lines. To assess the effects of differentiation agents and cytokines on ganglioside biosynthesis in leukemic lymphoblasts, CEM-2 cells were cultured with $^{14}$C-galactose and $^{14}$C-glucosamine in the presence or absence of phorbol myristate acetate (PMA), interleukin 2 (IL-2) and interferons (IF), either alone or in combination. Cells were harvested, and radiolabeled gangliosides were isolated from lipid extracts. Biosynthesis was quantitated by scintillation counting and densitometry of autoradiographs of thin layer chromatograms. After 3 days of culture, PMA increased total ganglioside synthesis 2.5 times relative to controls, and PMA combined with either IL-2 or IF- enhanced synthesis 3-4 times. IL-2 and IF- together with PMA resulted in additive effects. IF- could not replace IF- , and IL-2 or IF- did not increase total synthesis without PMA. Results of incorporation of radiolabel into individual gangliosides showed that PMA in the presence or absence of cytokines increased the proportion of $GM_3$ synthesized, and the absolute amounts of $GD_3$ synthesized increased due to increased total synthesis. IL-2 and IF- in combination also altered biosynthesis of individual gangliosides. The results indicate that ganglioside biosynthesis in T-ALL blasts can be modulated with differentiation agents and/or cytokines, implicating their potential use in altering ganglioside surface expression in these cells.

**126**

CASTANOSPERMINE, AN INHIBITOR OF GLUCOSIDASE-I, RESTORES CD4 AND CD8 POSITIVE LYMPHOCYTE FREQUENCY AND FUNCTION IN FRIEND LEUKEMIA VIRUS INFECTED MICE. Terry L. Bowlin, Prasad S. Sunkara and Albert Sjoerdsma. Merrell Dow Research Institute, Cincinnati, Ohio 45215.

Cell mediated immunity has been shown to be critical for the effective elimination of many types of viruses. We, and others, have recently established that castanospermine (CAST), an inhibitor of glucosidase-I during glycoprotein processing, has antiretroviral activity in vitro and in vivo. The objective of the present investigation was to examine the effect of CAST on T lymphocyte frequency and function in Friend leukemia virus (FLV) infected mice. FLV was injected (i.v.) on day 0 and CAST (50 mg/kg) was administered orally, q.i.d. Splenic lymphocytes were isolated from individual (n=7) uninfected control mice, FLV infected and CAST treated FLV infected mice on day 14. Cells were then labeled with fluorescein conjugated anti-CD4 and anti-CD8 monoclonal antibodies, and analyzed by flow cytometry. FLV infected mice exhibited a 31% and 51% decrease in CD4 and CD8 positive lymphocytes, respectively, compared with uninfected control mice. CAST administration completely returned CD4 and CD8 frequencies to those levels seen in uninfected control mice. Concomitant with the decrease in T cell number in FLV infected mice was a decreased proliferative response ([$^3$H]-TdR incorporation) to the T cell mitogen concanavalin A (Con A). CAST restored the T cell mitogenic response to normal levels in FLV infected mice. T cell proliferation is dependent upon the endogenous production of the CD4 lymphocyte product interleukin 2 (IL-2). Con A stimulated lymphocytes isolated from FLV infected mice exhibited greater than a 50% decrease in IL-2 activity. IL-2 production was returned to normal levels in CAST treated mice. The effect of CAST on T cell frequency, T cell proliferation and IL-2 production were all dose-dependent. These data indicate that CAST can not only reverse the effects of FLV infection on T cell frequency and function but also will not impair T cell function. Therefore, inhibitors of glucosidase-I, such as CAST, may be useful antiretroviral agents without the immunosuppressive side effects associated with other chemotherapeutic drugs.

127

CASTANOSPERMINE POTENTIATES ALLOANTIGEN INDUCED CYTOLYTIC T LYMPHOCYTE GENERATION FROM FRIEND LEUKEMIA VIRUS INFECTED MICE *IN VITRO*. Terry L. Bowlin and Christopher M. Roberts. Merrell Dow Research Institute, Cincinnati, Ohio 45215

Cytolytic T lymphocyte (CTL) activation is an important immune defense in a virally infected host. We, and others, have recently shown that inhibitors of glycoprotein processing can alter T cell responses to antigen and mitogens. The objective of the present investigation was to examine the effect of castanospermine (CAST), an inhibitor of glucosidase I during N-linked glycoprotein processing, on CTL induction from Friend leukemia virus (FLV) infected mice. CTL were generated in a one-way mixed lymphocyte reaction contained FLV infected Swiss splenic lymphocytes as responders and irradiated (2000R) DBA/2 splenic lymphocytes as stimulators. CAST (10 µg/ml) was added at the initiation of cell culture and was present throughout the five-day incubation period. Allospecific CTL generation from FLV infected mice was reduced greater than 50%, compared with uninfected controls. CAST increased CTL induction from FLV infected mice, in a dose-dependent manner, greater than two-fold. Optimal CTL induction requires expansion of the allospecific lymphocyte population. The effect of CAST on cell proliferation ([$^3$H]-TdR incorporation) was examined at 48 hr of culture. CAST (10 µg/ml) treatment increased cell proliferation by 2-3 fold. CTL proliferation is dependent upon the endogenous production of the T cell product interleukin 2 (IL 2). Supernatant fluids from MLR containing FLV infected splenocytes were examined for IL 2 activity. IL 2 activity was increased 2-3 fold in those cultures containing CAST, compared with untreated controls. These data indicate that CAST can augment alloantigen induced T cell proliferation, IL 2 production and cytolytic activity *in vitro* in lymphocytes isolated from FLV infected mice. Therefore, inhibitors of glycoprotein processing may be useful in potentiating antigen recognition by retroviral infected hosts.

# Enzymatic Degradation and Storage Diseases

## 128

α-L-FUCOSIDASE IN FUCOSIDOSIS. R.A. DiCioccio[1] and Bruce A. Gordon[2]. [1]Dept. of Gyn. Oncology, Roswell Park Memorial Institute, Buffalo, NY, [2]Ministry of Community and Social Services, London, Ont., Canada.

Fucosidosis is an autosomal recessive lysosomal storage disease due to a deficiency of fucosidase activity in tissues and body fluids. It was reported that the mutation(s) causing fucosidosis in two siblings and two unrelated patients affected the quantity, catalytic activity, and secretion of fucosidase protein as expressed by lymphoid cells (Biochem. Genet. 27:279-290, 1989). Lymphoid cell cultures from these patients contained reduced amounts of catalytically inefficient fucosidase that was hypersecreted. Here, it is reported that a fifth unrelated patient (JH) exhibits the same pattern of expression of fucosidase. Exponentially growing JH lymphoid cell cultures had 16-fold less extracellular fucosidase protein and 72-fold less intracellular fucosidase protein with negligible catalytic activity as compared to the mean of 19 control cultures. The percentage of total fucosidase protein released extracellularly by JH cells was 71% as compared to 35% ± 9% for control cells. During a 1.5 h pulse with $^{35}$S-methionine, fucosidase was synthesized by JH cells as an intracellular doublet with Mr of 58,000 and 56,000 and by control cells as an intracellular form with Mr = 58,000. During a subsequent 21 h chase with methionine, JH fucosidase was entirely processed to extracellular forms with Mr of 58,000 and 60,000. In contrast, only 25%-30% of control enzyme was processed to an extracellular form (Mr = 62,000) with the remainder retained intracellularly (Mr = 60,000). Treatment of intracellular and extracellular enzymes with N-glycanase revealed polypeptide chains with Mr of 52,000 and 54,000 for JH fucosidase and a single polypeptide chain with Mr = 52,000 for control α-L-fucosidase. The doublet of fucosidase in JH cultures may represent expression of two distinct allelic forms of mutant fucosidase or may result from abnormal post-translational processing of fucosidase. (Supported by DK32161).

## 129

ACCUMULATION OF FUCOSE-LABELED STORAGE PRODUCT IN FUCOSIDOSIS LYMPHOID CELL LINES. M.M.Klinger[1] and R.A.DiCioccio[2]. [1]Biochemistry Department, Southern Research Institute, Birmingham, AL 35255 and [2]Dept. of Gynecologic Oncology, Roswell Park Memorial Institute, Buffalo, NY.

The deficiency of α-L-fucosidase in patients with fucosidosis results in the accumulation of fucose-containing glycopeptides, oligosaccharides, and glycolipids in various tissues. Previous studies have employed nontransformed skin fibroblasts from fucosidosis patients to study alterations in the metabolism of fucoconjugates. Unlike skin fibroblasts, Epstein Barr virus-transformed lymphoid cell lines have an unlimited life span in culture, grow in suspension culture which facilitates passaging, and can be cultured in large quantities to facilitate biochemical investigations. Exponentially growing lymphoid cell cultures from fucosidosis patients are devoid of detectable α-L-fucosidase activity (R.DiCioccio, abstracts, Soc. for Complex Carbohydrates, 1988). We report here that [$^3$H]fucose-labeled fucosidosis lymphoid cells accumulate a low molecular weight product to a level several times greater than lymphoid cells derived from healthy individuals. The product has the same thin layer chromatographic mobility and gel filtration elution profile as fucosyl-N-acetylglucosaminyl-asparagine, a compound which accumulates in the urine of fucosidosis patients and in cultured fucosidosis fibroblasts. These results suggest that fucosidosis lymphoid cell lines may provide a useful model system for studying defects in fucoconjugate metabolism. We also observed that neither the fucosidosis nor the normal lymphoid cells synthesize detectable levels of glucosyl-fucosyl-threonine, a compound produced by a wide variety of mammalian fibroblast and epithelial cell lines grown on glass or plastic substrates (Klinger et al., J. Biol. Chem. 256:7932, 1981; Steiner et al., Cancer Res. 43:2628, 1983). The absence of this compound in cells grown in suspension suggests that it may play a role in cell-substrate attachment. (Supported by S07 RR05676 and DK32161).

**130**

α-L-FUCOSIDASE IN I-CELL DISEASE (ICD) AND PSUEDO-HURLER POLYDYSTROPHY (PHP) LYMPHOID CELLS. R.A. DiCioccio[1] and A.L. Miller[2]. [1]Dept. of Gyn. Oncology, Roswell Park Memorial Institute, Buffalo, NY, [2]Dept. of Neurosciences, UCSD, La Jolla, CA.

It was previously reported (Biochem. J. 248:151, 1987 and J. Cell Bio. 107 #1934, P. 341 A, 1988) that ICD and PHP lymphoid cells had nearly normal intracellular and intralysosomal activities of several lysosomal acid hydrolases despite a deficiency of N-acetylglucosaminylphosphotransferase. These results suggested that lymphoid cells may provide a unique system to investigate alternate mechanisms for targeting newly synthesized acid hydrolases to lysosomes. The biosynthesis, processing, and secretion of α-L-fucosidase in ICD and PHP lymphoid cells was used as a model system to study the existence of such mechanisms. Levels of intracellular fucosidase protein in exponentially growing ICD or PHP lymphoid cultures were comparable to the mean of 19 normal control cultures but levels of extracellular fucosidase protein in ICD or PHP cultures were 4.5-fold greater. The rates of synthesis of α-L-fucosidase protein by ICD or PHP cultures were 3-fold greater than normal control cultures while the rates of secretion were 7-fold greater. During a 1.5 h pulse with $^{35}$S-methionine, fucosidase was synthesized by ICD, PHP, and control cultures as an intracellular form (Mr = 58,000). Companion cultures chased with methionine from 2 h - 21 h processed the enzyme to an intracellular form (Mr = 60,000) and an extracellular form (Mr = 62,000). All enzyme forms were glycoproteins with polypeptide chains of Mr = 52,000. Treatment with endoglycosidase H indicated that newly made intracellular enzyme contained high mannose sugar chains and processed intracellular and extracellular enzyme contained complex sugar chains. Additional investigations of structural details and subcellular localization of fucosidase are currently under study to explain the maintenance of normal levels of fucosidase in ICD and PHP lymphoid cells.
(Supported by DK32161 and NS12138).

**131**

STUDIES ON THE DEGRADATION OF GLYCOSPHINGOLIPIDS BY A SOLUBLE CERAMIDE GLYCANASE FROM RABBIT MAMMARY TISSUES. C. Westervelt, J.W. Hawes, K.K. Das, M. Basu, M.J. Beuter, A. Shukla and S. Basu, Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556

A ceramide glycanase (CGase) activity has been detected in a soluble supernatant (100,000 xg) isolated from a rabbit mammary tissue homogenate that catalyzes the hydrolysis of [6-3H]GgOse4Cer to [6-3H]GgOse4 and ceramide. The reaction was stimulated in the presence of taurodeoxycholate (1.0 mg/ml). A broad range of pH optima (between 4.5-5) in citrate buffer was observed. However, the ceramide glycanase from earthworm shows a pH optimum of 4.0. During regular assay procedures [3H]GgOse4([6-3H]Galβ1-3-GalNAcβ1-4Galβ1-4Glc) was partitioned on the upper layer and characterized by silica gel G TLC with developing solvent, pyridine:ethylacetate:water (12:5:4).

The presence of CGase has been reported in non-mammalian eukaryotes (Li, Y.T., Ishikawa, Y. and Li, S.C. (1987) BBRC, 149, 167-172) and prokaryotes (Ito, M. and Yamagata, Y. (1986) J.Biol.Chem., 261, 14278-14282). To date, no such activity has been reported in a mammalian system. However, the above results suggest the possibility of a CGase activity as the source of glycosphingolipid (GSL)-type oligosaccharides in mammalian milk. The specificity of CGase towards GSLs might control the composition of oligosaccharides in the milk of a specific animal and is under study at present.
(Sup: NIH Grants NS 18005-07; CA-14764; and CA-33751)

## 132

ISOLATION OF CERAMIDE-GLYCANASE FROM THE EARTHWORM. B. Zhou, S.-C. Li and Y.-T. Li Department of Biochemistry, Tulane University School of Medicine, New Orleans, LA 70112

We have previously reported the presence of a unique glycolipid-splitting enzyme, ceramide-glycanase (CG) in the leech (BBRC 141, 346, 1986) and the earthworm (EW) (BBRC 149, 167, 1987). CG detaches the glycan chains from various glycosphingolipids (GSLs) by cleaving the bond between the glycan chain and the ceramide in GSLs. Because of the easy availability of EW, we have undertaken the isolation and characterization of CG from this source.

Gutted EW (300 g, Lumbricus terrestris) was homogenized with 5 volumes of $H_2O$ and centrifuged to obtain 1400 ml of crude extract. To this solution, protamine sulfate (2 g/100 ml of $H_2O$) was added dropwise to obtain the concentration of 0.7 mg/ml. The precipitate was removed by centrifugation and the supernatant was brought to 60% saturation with solid ammonium sulfate. The precipitate which contained CG was collected by centrifugation, dissolved in a small volume of 0.05 M phosphate buffer, pH 7.0 and further purified by chromatography on Bio-Gel A-0.5m, octyl-Sepharose and ConA-Sepharose columns. Using this scheme, we achieved 1100-fold purification with 25% recovery. The optimal pH of the CG from the EW was found to be between 3.5 and 4.5 which is more acidic than the leech enzyme. Moreover, the molecular weight of CG from EW was found to be much smaller than that from the leech. It is also more resistant to the inhibition by $Cu^{2+}$ and $Ag^{+}$ than the leech enzyme. Among various bile salts, sodium cholate was most effective in stimulating the hydrolysis of GSLs by CG from EW. Trition X-100 is less effective than sodium cholate. This CG has a similar specificity to that of the leech CG in hydrolyzing various GSLs. In addition to GSLs, CG from EW also detached glycan chains from lyso-GSLs, alkyl-lactosides and lactosyl-diglyceride. Thus, this enzyme should be regarded as a glycolipid-glycanase or a glycolipid glycan-detaching enzyme. Among the various animal tissues examined CG was only found in annelids. The biological function of CG in annelids remains to be elucidated. (Supported by NSF DMB 8617033 and NIH NS 09626).

## 133

TRANSGLYCOSYLATION AND TRANSFER REACTION ACTIVITIES OF ENDO-α-N-ACETYL-D-GALACTOSAMINIDASE FROM *STREPTOCOCCUS PNEUMONIAE*. Richard M. Bardales and V.P. Bhavanandan, Department of Biological Chemistry, The M.S. Hershey Medical Center of The Pennsylvania State University, Hershey, PA 17033

We have previously reported the purification and characterization of an endo-α-N-acetyl-D-galactosaminidase which hydrolyzes the O-glycosidic linkage between Galβ1→3 GalNAcα- and serine or threonine in mucins and mucin-type glycoproteins. In recent studies we observed that treatment of asialoglycoproteins having Galβ1→3 GalNAc α→Ser/Thr linkages with enzyme preparations containing glycerol resulted in formation of non-reducing trisaccharides. Increasing the concentration of glycerol in the incubation mixture resulted in increased formation of the trisaccharides and when [$^{14}$C]-glycerol was included in the reaction mixture the product contained [$^{14}$C]-label. The structure of the main trisaccharide (~80%) was deduced to be Galβ1→3 GalNAc α1→1(3) glycerol by analysis of sugar composition and the results of exoglycosidase treatment and periodate oxidation. The ability of the endoglycosidase to catalyze transfer of Galβ1→3 GalNAc to various acceptors was also demonstrated by incubation of the enzyme with the disaccharide and the test compound. Tris (hydroxymethyl) aminomethane, p-nitrophenol, threonine, D-glucose, D-galactose and D-fucose were all effective acceptors of Gal→GalNAc. Our findings indicate that enzyme preparations free of glycerol and buffers not containing hydroxy compounds should be used to avoid spurious results. Since the disaccharide with the reducing end blocked will not yield color in the Morgan-Elson reaction, enzyme activity measurements in the presence of glycerol or other acceptors of transglycosylation would lead to underestimates. The potential for the use of this enzyme in the synthesis of glycosides is illustrated by our studies. [Supported by U.S. PHS grant CA 38797].

134

GLYCOSIDASES IN EHRLICH ASCITES TUMOR CELLS AND ASCITES FLUID: PURIFICATION AND SUBSTRATE SPECIFICITY OF α-GALACTOSIDASE AND α-N-ACETYLGALACTOSAMINIDASE. Fumio Yagi and Irwin J. Goldstein, Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109

Alpha-galactosidase and α-N-acetylgalactosaminidase were isolated from Ehrlich ascites tumor cells on ε-aminocaproylgalactosylamine-Sepharose. Alpha-galactosidase was purified 170,000-fold and was free of other glycosidase activities. Alpha-N-acetylgalactosaminidase was purified 160,000-fold but exhibited a weak α-galactosidase activity which appears to be inherent in this enzyme.

Substrate specificity of the α-galactosidase was investigated with 12 substrates, and compared with that of the corresponding coffee bean enzyme. The pH optimum of Ehrlich α-galactosidase centered near 4.5, irrespective of substrate, whereas the pH optimum of the coffee bean enzyme was dependent on the substrate. The pH optimum of coffee bean enzyme for PNP-α-Gal was 6.0, which is 2.0 pH units higher than that for other substrates. The reverse was found for α-N-acetylgalactosaminidase. The pH optimum for the hydrolysis of PNP-α-GalNAc was 3.6, lower than the pH 4.5, required for the hydrolysis of GalNAcα1,3Gal. Coffee bean α-galactosidase showed a relatively broad substrate specificity, suggesting that it is suited for processing many kinds of terminal α-galactosyl linkages. On the other hand, the substrate specificity of Ehrlich α-galactosidase appears to be quite narrow. This enzyme was highly active toward the terminal α-galactosyl linkages of Ehrlich cell surface glycoprotein and laminin, both of which have been established to possess Galα1,3Galβ1,4GlcNAcβ-trisaccharide sequences. The α-N-acetylgalactosaminidase was found to be active toward the human blood group A-disaccharide, A-trisaccharide and glycoproteins with A-active carbohydrate chains.

Supported by a grant (CA20424) from the National Cancer Institute, NIH.

135

**PURIFICATION AND PROPERTIES OF α-MANNOSIDASES FROM MUNG BEAN HYPOCOTYLS.** **Gur P. Kaushal, Irena Pastuszak and Alan D. Elbein, Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas.**

**The microsomal fraction from mung bean hypocotyls contains enzymatic activities of the glycoprotein processing mannosidase I and mannosidase II, and also of aryl-mannosidases. These enzymes were solubilized from the microsomal fraction using 1.5% Triton X-100. The mannosidase I that removes α1,2-linked mannose residues from [$^3$H]-Man$_9$GlcNAc was purified about 130-fold by conventional methods and also by affinity chromatography on mannan-Sepharose and mannosamine-Sepharose. The mannosidase II that removes both the terminal α1,3-linked and the terminal α-1,6-linked mannoses from GlcNAc-Man$_5$GlcNAc was purified to apparant homogeneity using conventional methods, as well as Concanavalin A-Sepharose and preparative gel electrophoresis. The various properties of these processing mannosidases were similar to those observed for the mammalian enzymes. During the purificiation of these processing enzymes, two peaks of arylmannosidase activity, capable of releasing mannose from p-nitrophenyl-α-D-mannoside, were observed on the DEAE cellulose columns. Both of these activities were purified to apparant homogeneity using conventional techniques followed by Concanavalin A-Sepharose chromatography. Although the 2 peaks of arylmannosidase activity were well separated from each other on DEAE-cellulose, phosphocellulose and hydroxyapatite columns and also by native gel electrophoresis, they showed the same molecular weight of 220 Kd on gel filtration on Sephacryl S-200, suggesting that they differ from each other by some charged groups on the protein. SDS gel electrophoresis under reducing conditions gave the same 4 subunits for each aryl-mannosidase. Both enzymes are sensitive to digestion by endoglucosaminidase H and both bind to Concanavalin A-Sepharose and are eluted with -methylmannose indicating that they are N-linked glycoproteins. The pH optima for both enzymes is between 4.2 and 4.5, and there is no requirement for metal ion for activity. Both enzymes are inhibited by swainsonine with 50% inhibition being observed at less than 0.5 umolar. Polyclonal antibodies raised against the first arylmannosidase cross react with the second enzyme. Further immunochemical studies are in progress. (Supported by NIH DK 21800 and the Robert A. Welch Foundation).**

## 136

DETECTION OF METALLOPROTEINASES IN REGIONS OF THE EMBRYONIC CHICK TIBIOTARSUS USING SDS-PAGE. A. Cole, K. Kuettner and T. Schmid. Rush Presbyterian-St. Luke's Medical Center, Chicago IL 60612 USA

During skeletal development, the marrow cavity forms as the cartilage anlagen is invaded by blood vessels. The vascular invasion is accompanied by degradation of cartilage matrix. We have investigated the production of proteases detectable in the culture medium conditioned by 4 different regions of embryonic chick tibiotarsus during the period of vascular invasion using SDS-PAGE on collagen, gelatin, proteoglycan (PG) and casein substrate gels. The 4 regions were 1) tarsus (epiphysis), 2) zones 1 + 2 (proliferating and some synthesizing cartilage), 3) zone 3 (hypertrophying cartilage + marrow cells) and 4) bony collar. The regions were cultured in serum-free DMEM. Samples (15 ul) of 24 hr conditioned media were analyzed on polyacrylamide gels copolymerized with 0.5 mg substrate (rat type I collagen, gelatin, chondroitin sulfate PG $A_1D_1$ or casein). The gels were incubated overnight at 37$^0$ C in the presence or absence of 0.5 mM APMA. The proteolytic activity detected on the gels was inhibited by EDTA and 1,10 phenanthroline but not by PMSF. Activity was detectable from all 4 regions on all 4 types of gels. Collagenolytic activity appeared as a doublet with a $M_r$ of 64K and 59K. In the presence of APMA, an additional doublet with a $M_r$ less than 45K was visible. Conditioned media from zone 3 + marrow differed from the other regions in that a smear was visible from high molecular weight to the 64K/59K doublet. Gelatinolytic activity was similar to the collagenolytic activity except that in all four regions a broad smear was present with an apparent $M_r$ of 120-170K. An additional band was also visible on the gelatin gel at $M_r$ of 80K. Only faint activity could be detected on the casein gels. The clearing on the casein gel was approximately 5X greater in zone 3 + marrow. This band migrated slightly faster than the doublet seen on collagen and gelatin when analyzed on a composite gel containing casein/gelatin. The detection of proteolytic activity on the PG gel was similar to the pattern seen on the casein gel. Slightly more enzyme was detected. In the zone 3 + marrow, a second band with an apparent $M_r$ of 50K was visible below the 57K band detected on the casein gel. The data indicate that all 4 regions of the embryonic chick tibiotarsus 1) contain detectable collagenolytic, gelatinolytic, caseinolytic and proteoglycan-lytic activity, 2) at least 3 different metalloproteases are present and 3) the concentration of some of the metalloproteases in the hypertrophic cartilage + marrow region appears to be elevated. This research was supported, in part, by AR-39239, Training Grant AR-07375 and BRSG.

## 137

GLYCOPEPTIDES SHED FROM TUMOR CELLS ARE DIGESTED BY HUMAN SERUM
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Glycopeptides or oligosaccharide fragments derived from antigens that are shed from tumor cells may block the interaction of cytotoxic immuno–effector cells with tumor cell targets. The release of low molecular weight glycoslyated material from tumor cells into the circulation may disrupt the receptor–mediated endocytosis system in hepatocytes, or prevent the binding of monoclonal antibodies to tumor cells or to circulating tumor antigens.

To determine if tumor cells shed low molecular weight glycopeptides in the presence of human serum, we radiolabeled colorectal and breast carcinoma cell lines with D–[$^{14}$C]glucosamine and D–[2–$^3$H]mannose and exposed the radiolabeled cells in culture to serum from cancer patients or from normal donors or to fresh medium for 1, 5, 12, or 24 hr. Cells were treated in log phase and in plateau phase. The culture fluids were removed from the cells and analyzed for their content of low molecular weight radiolabled glycosylated material by an HPLC gel filtration method designed for the rapid detection of glycopeptides.

Analysis of the radiolabeled components released from tumor cells in plateau phase showed that a 600 M, mannose–rich glycosylated component representing nearly 30% of the mannose radioactivity was released within 1 hr after the initiation of serum treatment. This factor was subsequently completely degraded during the next 12 hours of exposure to serum from a cancer patient. The rate of digestion of the factor was approximately 50% faster when normal serum was used. Comparable mannosyl components accounted for only 2% of total mannose radioactivity in log phase cells. Glucosamine–rich components in plateau phase cells exhibited a markedly different behavior occurring in negligible amounts after 1 hr of exposure and increasing to less than 15% of total glucosamine radioactivity after 24 hr of exposure. In log phase cells, glucosamine containing components released into exogenous serum represented nearly 30% of total cpm after 24 hr.

These studies have shown that separate mannose–rich and glucosamine–rich glycopeptides are shed in vitro into serum by live tumor cells. The mannosyl, but not the glucosaminyl components are subsequently degraded by contact with the serum. This process may be involved in the release of low molecular weight glycopeptides into the circulation and may play a role in the immunological "invisibility" of cancer cells.

# Glycosidase Inhibitors

## 138

IDENTIFICATION OF THE ACTIVE–SITE RESIDUE OF YEAST INVERTASE AFFINITY LABELED WITH CONDURITOL B EPOXIDE. V. Anthony Reddy and Frank Maley. Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY 12201.

Deglycosylated yeast invertase is irreversibly inactivated by conduritol B epoxide, an active–site directed reagent which structurally resembles the glucose moiety of the substrate sucrose. A double reciprocal plot of first order rate constant of inactivation ($K_{app}$) versus CBE (racemic mixture) yields a $K_d$ of 23 mM for enzyme–CBE complex, a value close to the $K_m$ for sucrose (25mM). Affinity labeling with [$^3$H]–CBE resulted in the incorporation of 0.78 residue of the label into the enzyme, suggesting modification of a single amino acid residue. Digestion of labeled invertase with pepsin followed by HPLC on a C8 column yielded two unequal radioactivity peaks. The larger radioactivity peak (2/3 of the counts) represented peptide I encompassing residues 20–26 in the amino–acid sequence. The smaller peak (1/3 of the counts) represented peptide II encompassing residues 1–26. Tryptic digestion of peptide II yielded labeled peptide III, located at residues 19–26 in invertase sequence. Sequence analysis of peptides I and III revealed no identifiable PTH–amino acid at residue 23. However radioactivity was released in this cycle, suggesting that Asp23 is the modified residue. Incubation of peptides I and III at pH 11.5 resulted in the release of the labeled conduritol moiety. Sequencing of the treated peptides now revealed the presence of PTH–Asp23, implicating Asp23 in the catalytic process. (Supported in part by grant CA44355 from the National Cancer Institute).

## 139

ACCUMULATION OF HIGH MANNOSE OLIGOSACCHARIDES IN HUMAN MONONUCLEAR LEUKOCYTES BY SWAINSONINE.

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Treatment of mammalian cells with swainsonine, a known inhibitor of mannosidase II involved in asparagine-linked glycoprotein processing, results in the accumulation of hybrid type oligosaccharide containing glycoproteins. We have recently shown that swainsonine, can augment lymphokine-activated killer (LAK) cell induction at sub-optimal doses of interleukin 2 (IL-2) (Cancer Research 49 August 1989). The amount of swainsonine needed to increase LAK activity was 50-100 fold higher (10 µg/ml) than required to completely inhibit mannosidase-II (0.1-0.2 µg/ml). Therefore, we wanted to investigate why such a high concentration of swainsonine was necessary to obtain the increase in LAK activity. Human mononuclear lymphocytes (MNL) treated with swainsonine (0.1-10 µg/ml) resulted in 2-4 times higher incorporation of 2-[$^3$H]Mannose into glycopeptides as compared to control. On chromatographic analyses of glycopeptides on a Bio-gel P-4 column, a new glycopeptide peak appeared at 10 µg/ml of swainsonine. This peak contained 36% of the radioactivity incorporated into glycopeptide pool with an apparent molecular weight of 1800 daltons. The MNL glycopeptide fraction was resistant to endoglycosidase H, endoglycosidase F, O-glycanase and N-glycanase treatments. However, these glycopeptides were susceptible to cleavage by Jack bean α-mannosidase and were bound more than 90% to concanavalin A-sephrose. A similar peak was observed in 2-[$^3$H]Mannose labelled glycopeptides from mouse $B_{16}$ melanoma and Baby Hamster Kidney (BHK) cells following swainsonine (10 µg/ml) treatment. These results indicate that at the higher concentrations, swainsonine may lead to accumulation of high mannose rather than expected hybrid type of oligosaccharides. Further analysis of this unusual glycopeptide is in progress.

**140**

**THE EFFECT OF CASTANOSPERMINE AND SWAINSONINE ON INTESTINAL SUCRASE.** Y.T. Pan, John Ghidoni and Alan D. Elbein, Departments of Biochemistry and Pathology, Univ. of Texas Health Science Center, San Antonio, Texas.

Castanospermine is an indolizidine alkaloid found in the seeds of the Australian tree, Castanospermum australe. Animals that eat these seeds suffer severe gastrointestinal upset, frequently leading to death. In order to determine the reason for this toxicity, we examined the effect of administration of castanospermine on the intestinal enzymes of mice and rats. Animals were given various amounts of castanospermine by intraperitoneal injection, every day for 3 to 4 days. At various times after injection, animals were sacrificed and the activities of various intestinal enzymes were determined, and compared to those of control animals. After three injections of castanospermine at a dose of about 200 ug/g body weight, there was no detectable sucrase or maltase activity in the intestinal extracts of these animals. Trehalase and ismaltase were also inhibited to some extent but much less so than the sucrase and maltase, while other glycosidases such as β-galactosidase and α-mannosidase were not affected. When the activities of these enzymes were examined over a time course of injection of castanospermine, it was found that the sucrase and maltase activities disappeared within 1 or 2 hours after the first injection, indicating that castanospermine was probably binding to the enzyme and inhibiting its activity. Interestingly enough, the injection of swainsonine into animals (40 ug/g body weight) also caused a loss of sucrase activity, but in this case it took up to 24 hours for the effect to become complete. It is not clear whether swainsonine affects the targeting of sucrase to the brush border, but it does cause a change in the N-linked oligosaccharide structure to that of hybrid types of structures. Castanospermine was also tested against the purified sucrase prepared from rat intestine. This alkaloid completely inhibited the sucrase at less than 1 umolar concentrations and this inhibition was of the competitive type. Sucrase was also very sensitive to inhibition by deoxynojirimycin and was also inhibited, but less strongly, by 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine and 6-epicastanospermine. Interestingly enough, swainsonine did not inhibit sucrase in vitro, even at high concentrations, although it does affect the activity in vivo as indicated above.

**141**

ISOLATION AND PARTIAL CHARACTERIZATION OF AN ELASTASE INHIBITOR FROM BOVINE NASAL CARTILAGE. Homandberg, G.A., Sawhney, B., *Tripier, D. and Kuettner, K.E. Department of Biochemistry, Rush-Presbyterian-St Luke's Medical Center, Chicago, Il 60612; *Hoechst Pharmaceutical Co., Frankfurt, West Germany.

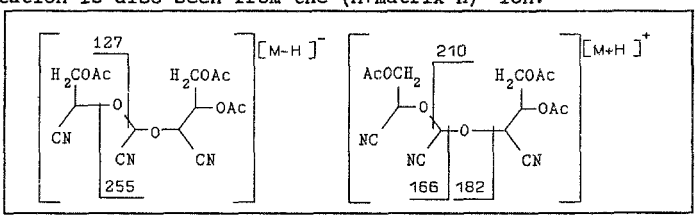
Leukocytic proteinases play a major role in pathogenesis of articular cartilage in arthritic diseases. AIF (anti-invasive factor) was originally described as an extract of bovine nasal septum cartilage that inhibited elastase, collagenase, other serine proteases, metallo-proteinases, endothelial cell growth and invasion of tumor cells. We report here partial characterization of this elastase inhibitor. Inhibitor was extracted from bovine nasal septum cartilage in 1 M NaCl, 50 mM Tris, pH 7.4. Extracts were subjected to Sephadex G-75 gel-filtration chromatography in guanidine HCl, resulting in a major peak containing proteoglycan of over 100 kD and a later small peak which contained two proteins of 15/17-kD. This smaller peak contained nearly all of the inhibitory activity. The relative amount of inhibitory material in crude extracts, was about 8% of the soluble extracted protein or about 0.008 % of the weight of nasal cartilage, and thus represents a high inhibitory potential in cartilage tissue. The doublet was resolved by subsequent Sephadex G-50 chromatography in 1 M GuHCl into two equally active fractions. The 17-kD inhibitor has an amino acid composition of 30% Asx+Glx and its Lys+Arg+His content of 14%. Ser was the major amino acid and accounted for 18% of the total residues. The isolated 17-kD showed less than 0.1% proteoglycan by weight using the DMBB method of detection. The inhibitor appeared to account for the major elastolytic and cathepsin G inhibitory properties of AIF and bound human sputum, human leukocytic and porcine pancreatic elastases with app Ki values of 0.2-0.6 uM. The app Ki estimated for inhibitor when assayed in the crude extract was 5 to 10 fold lower than when purified, a discrepancy still under investigation. The kinetic constants for association and dissociation confirm the 0.2 to 0.6 uM app Ki and suggest that the uM affinity is due to a relatively slow association step. The inhibitor bound sputum elastase maximally between pH 4 and 9 and at ionic strengths between 0.15 and 1 M NaCl. At greater than uM concentrations the inhibitor did not inhibit chymotrypsin, trypsin nor bacterial collagenase. The inhibitor could not be fragmented by high concentrations of proteases in the native, disulfide reduced, or SDS treated states. The isolated inhibitor, at 1 uM, slowed growth of bovine aortic endothelial cells similar to the effect observed with crude AIF. We have identified a similar size and specificity inhibitor in extracts of bovine articular cartilage. At present, we have been unable to identify the inhibitor, however its relatively high concentration in cartilage tissue suggests that it originates from structural cartilage protein. We are continuing to investigate the mode of action and significance of this elastase inhibitor in cartilage. This work was supported by NIH grant 1-P50-AR39239.

# Analytical Techniques

## 142

SEQUENCE ANALYSIS OF COMPLEX CARBOHYDRATES VIA PERACETYLATED POLYNITRILE DERIVATIVES. R.S. PAPPAS, B.J. SWEETMAN, S. RAY, C.G. HELLERQVIST. VANDERBILT UNIVERSITY SCHOOL OF MEDICINE, DEPARTMENTS OF BIOCHEMISTRY AND PHARMACOLOGY, NASHVILLE TN 37232.

We have reported previously a method for the determination of sequence of sugar residues in poly- and oligosaccharides based on their linkages, using periodate oxidation, borodeuteride reduction and peracetylation as the principle derivatization scheme (1). An expert system GLYCOSPEC utilizing sugar, linkage, and f.a.b.-m.s. data from the derivatized material will provide the user with most probable structures. The very simple fragmentation pattern observed encouraged investigation of derivatives with alternative functional groups, which could stabilize the primary fragment ions and thereby enhance the sensitivity. We now report on studies using peracetylated polynitrile derivatives obtained from periodate oxidized oligosaccharides. Purified samples were analyzed by positive and negative f.a.b.-m.s. utilizing 3-nitro-benzylalcohol as the matrix. The schematics illustrate fragmentation typical of these derivatives. In addition to the anticipated fragmentation, negative ion f.a.b.-m.s., yields $(M-H)^-$ with loss of multiples of $CH_2CN$ and $COCH_2$. This fragmentation is also seen from the $(M+matrix-H)^-$ ion.

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## 143

ANALYSIS OF STRUCTURE-ELUTION VOLUME RELATIONSHIP IN HIGH PERFORMANCE ANION EXCHANGE CHROMATOGRAPHY OF OLIGOSACCHARIDES --- PARAMETERIZATION OF CONTRIBUTING MONOSACCHARIDE UNITS. K. Kakehi, B.I.C. Lee, and Y.C. Lee. Dept. of Biol., JOHNS HOPKINS UNIVERSITY, Baltimore, MD 21218

One of the most powerful methods for analysis of oligosaccharides is high performance anion exchange chromatography. Its superb resolution enables it to "map" oligosaccharides in glycoconjugates. The "mapping" of oligosaccharides can be achieved by direct comparison with known standards, but it is impractical for average investigators to obtain all the oligosaccharides in pure form for this purpose. Another approach is to establish structure-elution volume relationship so that only a minimal number of standards are required for assignment of oligosacchride structure. We have analyzed the existing chromatographic data of a number of oligosaccharides by assuming the principle of additivity, i.e., the elution volume of a given oligosaccharide is the sum of the contribution of each of the constituent monosaccharide units. The monosaccharide unit in this case is also characterized by its anomeric configuration as well as its position of attachment and its localization in the branches. Each of the monosaccharide units was assigned a parameter, and the parametric values were solved by iteration using a commercial program, EUREKA (Borland International), until the parametric values became constant. Alternatively calculation was done with a multiple regression program written in Turbo PASCAL. The final parametric values for each monosacchride unit are such that the calculated elution volumes of the oligosaccharides were within 2.5% of the observed values. Inclusion of more data in this scheme of calculation should lead to a prediction of elution volume from structure or a prediction of structure from elution volume. This will greatly enhance the "mapping" strategy of structural determination of oligosaccharides in glycoconjugates.

## 144

**CHARACTERIZATION OF OLIGOSACCHARIDES AND N-LINKED GLYCANS BY CHEMICAL DERIVATIZATION, SUPERCRITICAL FLUID CHROMATOGRAPHY MASS SPECTROMETRY.**

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SFC-MS brings new opportunities for improving carbohydrate analytical chemistry by providing component separation in a mobile phase with diminished resistance to mass transfer (compared to $H_2O$) which provides enhanced column resolution; and, increased solvating power (compared to gas) for low temperature high molecular weight chromatography. These factors combine to provide excellent oligomer separations, but equally as important, this single-phase fluid is easily eliminated upon release of pressure markedly diminishing the problems of MS interfacing. Beyond improvements in specificity, SFC-MS will also provide advancements in sensitivity by applying well established negative ion chemical ionization (NCI) MS methodology using derivatization with electron capture groups. We have demonstrated both improved component resolution by capillary SFC and femtomole sensitivity using a bimodal reducing-end derivative pentafluorobenzyl aminobenzoate (1). This approach is now being used to profile N-linked glycans following enzymatic deglycosylation, derivatization, and SFC-NCI-MS (2). Additional steps of chemical modification (3-5) to introduce greater molecular specificity are under active investigation.

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## 145

**RAPID N-LINKED OLIGOSACCHARIDE MAPPING OF GLYCOPROTEINS USING A CARBOHYDRATE ANALYZER.**

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We have developed chromatographic conditions for the separation of neutral (high mannose and complex type) and sialic acid containing oligosaccharides released with endo-ß-N-acetylglucosaminidase F (Endo F) and peptide N-glycosidase (PNGase/glycanyl-amidase) F respectively on a carbohydrate analyzer (Dionex Bio LC). All the carbohydrate separations were carried out on a polymeric pellicular anion exchange column HPIC-AS6/CarboPac PA-1 (Dionex) using only two eluants, namely 0.5M NaOH and 3% acetic acid-NaOH pH 5.5, which were mixed with water to generate various gradients. Quantitative detection of carbohydrates was carried out with an electrochemical pulsed amperometric detector and a gold electrode working at the following potentials: $E_1$=0.01V($t_1$=0.3s); $E_2$=0.7V($t_2$=0.12s); $E_3$=-0.3V($t_3$=0.3s). The detector reference electrode cavity was packed with a fine powder of $BaCO_3$ wetted with 0.5M NaOH for stabilizing the pH effectively. Column effluent was mixed 1:1 with a solution consisting of 20ml of 50% NaOH and 5g of Na acetate per liter prior to the detector. These conditions were necessary to obtain steady baselines with suitable sensitivity ( 25 pmol at 0.1-0.3 µA output) in separations employing a variety of gradient programs.

Sialylated oligosaccharides were separated into groups based on charge content at pH 5.5 using a Na acetate gradient. Sialylated oligosaccharides obtained from human transferrin, bovine fetuin and human $\alpha_1$-acid glycoprotein gave multiple peaks in each group at pH 5.5 presumably due to a significant contribution of carbohydrate size to the separation. Baseline separations were obtained with neutral oligosaccharide types using NaOH and Na acetate gradients but mixtures of high mannose and complex types were poorly resolved. The high mannose peaks were eliminated specifically by digesting with α-mannosidase. PNGase F and Endo F released oligosaccharides from porcine thyroglobulin were separated within 60 min to obtain an "oligosaccharide map" in which oligosaccharides eluted in the order of neutral , mono, di-, tri- and tetra- sialylated species based both on charge and size.

146

## A MULTI-MODE CHROMATOGRAPHIC APPROACH FOR THE PURIFICATION OF OLIGOSACCHARIDES FROM CHITIN.

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In elucidating oligosaccharide structures, identification of an unknown structure based on chromatographic retention time alone, is not convincing because of the immense number of possible structural isomers which may co-elute with the compound of interest. Unequivocal structure determination of oligosaccharides requires the use of high field NMR and/or FAB-mass spectrometry. One drawback of these techniques is that they require a large quantity of a homogeneous sample. High-performance liquid chromatography (HPLC) has been used to purify large quantities of oligosaccharides in high purity from complex mixtures. Described in this paper is a multi-mode chromatographic approach, using stable polymeric columns, which demonstrate the usefulness of HPLC in the isolation and purification of oligosaccharide fragments from chitin.

147

## CHARACTERIZATION OF GLYCOPROTEINS BY HPLC - PEPTIDE MAPPING AND ANALYSIS OF SITE SPECIFIC GLYCOSYLATION.

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Once purified to homogeneity, recombinantly produced glycoproteins are characterized by a number of methodologies (peptide mapping, amino acid analysis, sequencing, and carbohydrate profiling) in order to ensure batch to batch reproducibility. Two of these methodologies, namely peptide mapping and the profiling of site specific glycosylation have been performed using new column chemistries and instrumentation. A tryptic digest of fetuin was analyzed by HPLC before and after treatment with N-Glycanase® using the Waters Peptide Analyzer. Carbohydrate containing peptides were preparatively isolated by HPLC and treated with N-Glycanase to release the N-linked oligosaccharides. Following removal, the oligosaccharides were fractionated according to the degree of sialylation. This separation, coupled with high sensitivity detection, has provided a profile of the oligosaccharides attached to a specific glycopeptide.

**148**

INVESTIGATIONS ON THE USE OF CAPILLARY ELECTROPHORESIS FOR THE STUDY OF CARBOHYDRATES

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Capillary Electrophoresis has recently appeared on the scene and is quickly finding its way into the study of biological molecules. In recent months much has appeared on the use of this method for separating peptides, and nucleic acids as well as a host of other biological and non-biological molecules. We have investigated approaches to the electrophoretic separation of carbohydrates based on techniques commonly used in chromatographic methods, namely high pH, borate complexes and derivatives.

**149**

VOLTAMMETRIC BEHAVIOR OF MODEL CARBOHYDRATES IN BASIC SOLUTIONS AT GOLD ELECTRODES, Richard W. Andrews & Richard M. King, Waters Chromatography Division, Millipore Corp., Milford, MA 01730

The chromatographic detection of carbohydrates by triple pulse amperometry is generally performed in strongly basic solutions with gold working electrodes. The literature contains a number of recommended programs for the detection of simple sugars, but provides little guidance in the selection of detection potentials for complex carbohydrates. The cyclic voltammetry of model sugars in basic solutions is presented as a guide to the appropriate selection of detection potentials. The voltammetry of both the model sugars and gold electrode are examined as a function of solution pH. An optimization procedure for the detection of carbohydrates is presented. The procedure accounts for differences in both solution pH and the reference electrode in use. The application of the procedure to chromatographic detection of carbohydrates is demonstrated.

150

SINGLE-STEP EXOGLYCOSIDASE ANALYSIS OF OLIGOSACCHARIDES. R. R. Townsend and M. R. Hardy. Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94143-0446 and Dionex Corporation, Sunnyvale CA, 94088-3603

Sequential exoglycosidase analysis of purified oligosaccharides is often used to identify terminal monosaccharide units, establish the anomerity of sugar linkage, and to determine linkage position of the released monosaccharides. Additional structural information can be obtained from quantification of the released monosaccharides. This approach often requires multiple separations and/or derivitizations with the consequent difficulty of obtaining quantitative information relating to the original sample. We have applied high-pH anion-exchange chromatography with pulsed amperometric detection to the sequential exoglycosidase analysis of oligosaccharides. In a single chromatographic step, we could separate the relevant monosaccharides and the asialo- and agalacto-N-linked oligosaccharides of the bi, tri- and tetra-series (with and without a core $\alpha(1,6)$-linked Fuc). Exoglycosidase digests containing neuraminidase, $\beta$-galactosidase, $\beta$-hexosaminidase, singly or in combination, were successfully analyzed by injecting the unprocessed digest into the chromatograph. The course of the enzymatic reactions were assessed by quantifying the amount of monosaccharides released and the change in retention time of the starting oligosaccharide. The completeness of release of terminal sugars was determined by monosaccharide analysis of the retention-time shifted oligosaccharides followed by additional enzyme treatments, as necessary.

151

A SENSITIVE METHOD FOR QUANTITATING GANGLIOSIDES OF THE GANGLIO SERIES DIRECTLY ON THIN-LAYER CHROMATOGRAMS. L.D. Cambron and K.C. Leskawa, Dept. Anatomical Sciences and Neurobiology, University of Louisville, Louisville, KY 40292.

The fact that ganglioside GM1 is the cell surface receptor for cholera toxin has been used to develop a quantitative assay using plastic multiwell plates (Wu and Ledeen, Analyt. Biochem., 173:368, 1988). The difficulty with this procedure is that adherence of gangliosides to the plastic wells requires demanding techniques, and at best only 40-48% of the added compounds actually attach. These problems were overcome by employing an overlay assay, whereby gangliosides were separated on nano-TLC plates, 'fixed' with polyisobutylmethacrylate, and overlayed with cholera toxin subunit B conjugated to horseradish peroxidase. Color development was performed with 4-chloronaphthol. Absolute quantitation was realized using a Shimadzu CS-9000 integrating spectrodensitometer, scanning at 580 nm. A correlation coefficient of 0.81 was obtained in a linear range of detection from $10^{-13}$ to $10^{-16}$ moles. Statistical analysis revealed good reproducibility and over 99% of the added gangliosides remained with the chromatogram during all overlay and washing procedures. This range of quantitation is several orders of magnitude lower than standard HPLC techniques which require derivatization. This overlay method was compared to standard chemical visualization of thin-layer chromatograms, using resorcinol. In this case, a correlation coefficient of 0.96 was found in the nanomole range with maximum sensitivity of only $10^{-10}$ moles. Following separation of a mixture of complex gangliosides, the TLC plate can be incubated with neuraminidase from Clostridium perfringens, reducing the structures to GM1, which can then quantitated by this method. (Supported by NIH grant NS 21057)

152

COLORIMETRIC DETERMINATION OF O-GLYCOSIDICALLY LINKED SACCHARIDES IN MUCIN GLYCOPROTEINS. V.P. Bhavanandan and M. Sheykhnazari, Dept. Biol. Chem., Hershey Med. Ctr., PA State U., Hershey, PA 17033

Even though there is great interest in understanding the structure and function of mucins, progress has been slow primarily due to difficulties in purifying these very large size molecules which appear to avidly bind other components of mucus. During the various purification steps, the fractions containing mucins are typically detected by assaying for neutral sugars (hexoses) or sialic acid. However, this is not satisfactory since these saccharides are also components non-mucin glycoproteins. The purpose of this study was to develop a colorimetric assay for the detection of mucins and glycoproteins with O-glycosidically linked saccharides in the presence of glycoproteins containing N-glycosidically saccharides and proteoglycans. The method developed combines the susceptibility of N-acetylgalactosamine terminating O-glycosidically linked saccharides to β-elimination with the Morgan-Elson reaction for N-acetylhexosamines with free reducing end. Glycoprotein samples are first heated with sodium borate buffer, pH 9.1 at 100° for 2 hours to release O-glycosidically linked saccharides and convert the exposed terminal GalNAc to chromogenic substances. The color development is then achieved by the Morgan-Elson reaction by treatment with p-dimethyl amino benzaldehyde at 37° for 20 min. All mucin and mucin-type glycoprotein samples but none of the serum-type glycoproteins tested resulted in characteristic color production. Various mucins gave linear responses in the range of 5 to 200 μg and the method was also adapted to the microscale involving the use of plastic microtiter plates. The micro method in which the volumes of sample and reagents were scaled down 5-fold was very convenient for monitoring the column chromatographic and density gradient centrifugation purification of mucins. The utility of the method for the specific detection of mucin in chromatographic column effluents was demonstrated. [Supported by US PAS grant HL 42651].

# Structure and Conformation

## 153

THE ASPARAGINE LINKED SUGAR CHAINS OF RECOMBINANT HUMAN TISSUE PLASMINOGEN ACTIVATOR PRODUCED IN CHINESE HAMSTER OVARY CELLS.

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Tissue plasminogen activator (t-PA) is a serine protease which plays a key role in the *in vivo* lysis of fibrin clots by the activation of plasminogen to plasmin. The cloning and expression of t-PA has led to its development as a therapeutic agent for thrombolysis in myocardial infarction.

The primary structure of human t-PA has been determined by both protein and cDNA sequence analysis and indicates the presence of four potential Asn-linked glycosylation sites. Three of these sites at Asn-117, Asn-184, and Asn-448 have been shown to be glycosylated.

We have analyzed the Asn-linked sugar chains of recombinant human t-PA produced in Chinese hamster ovary (CHO) cells. The Asn-linked sugar chains of recombinant human t-PA were released by treatment with N-Glycanase®enzyme. The released sugar chains were isolated, labeled by reduction with sodium borotritide and analyzed by a combination of hplc and exoglycosidase digestions.

The results showed the presence of both high mannose (30%) and complex type (70%) oligosaccharides. The high mannose type oligosaccharides consisted of $Man_5GlcNAc_2$, $Man_6GlcNAc_2$ and $Man_7GlcNAc_2$. The majority of the complex oligosaccharides were biantennary chains with lesser amounts of triantennary and tetraantennary structures. All of the complex oligosaccharides had fucose attached to the proximal N-acetylglucosamine.

## 154

**MOLECULAR MODELING OF O-LINKED GLYCOPEPTIDES FROM OVINE SUBMAXILLARY MUCIN**

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We have recently shown that the O-linked carbohydrate side chains of mucous glycoproteins are responsible for stiffening and expanding the mucin peptide core [Shogren et al., Biochemistry,28, 5525 (1989)]. For example the removal of the NeuNAc (2-6) GalNAc- disaccharide side chains from ovine submaxillary mucin (OSM) results in a nearly 3 fold contraction of the mucin chain dimensions to those typical of denatured globular proteins. A simple rotational isomeric state analysis of the conformation of the mucin peptide core suggests that glycosylation significantly restricts the peptide core dihedral angles compared to a random coil peptide (Shogren et al. 1989). These observations together with those of earlier studies suggest that steric interactions of the O-linked GalNAc residue with the peptide core are primarily responsible for the expanded mucin structure and that these perturbations extend to the nonglycosylated amino acid residues.

To further examine the origins of the expanded mucin conformation empirical and semi-empirical molecular modeling calculations have been initiated for a series of OSM model glycopeptides. The calculations consist of molecular mechanics and molecular dynamics modeling using the AMBER parameterization supplemented with α-NeuNAc(2-6)-α-GalNAc- specific parameters determined from model fragments by molecular orbital calculations. The objectives of these calculations are to elucidate specific carbohydrate-peptide interactions that will account for the observed expanded conformation of the mucin molecule. Of primary interest is the determination of the conformation(s) and intramolecular interactions of glycopeptides containing adjacent, presumably, glycoslyated Ser/Thr residues. Such clusters of Ser/Thr residues are common features in the amino acid sequences of the mucins described to date and thus may be required for the expansion of the mucin molecule upon glycosylation.

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155

**A NEW APPROACH FOR CHEMICALLY DEGLYCOSYLATING MUCOUS GLYCOPROTEINS**

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The chemical deglycosylation of mucous glycoproteins is most commonly performed using the trifluoromethanesulfonic acid (TFMSA) method of Edge et al. [Anal. Biochem. 118, 131 (1981)]. Although the relatively mild reaction conditions described can remove much of the mucin carbohydrate, removal of the peptide linked GalNAc residues requires longer times and higher temperatures. For example, the O-linked GalNAc residues of ovine submaxillary mucin (OSM) are nearly quantitatively retained following TFMSA treatment for 5 hr at $0^{o}$ C while treatment for 7 hr at $25^{o}$ removes 80-90% of the GalNAc but reduces the molecular weight of the apo mucin to about 20,000 [Shogren et al., Biochemistry 28, 5525 (1989)].

In studies directed towards modifying the carbohydrate structure of OSM, we found that periodate oxidation caused the side chains of OSM to become unusually labile at elevated pH. An examination of the structure expected for the $NaIO_4$ oxidation product indicates the potential for a reverse $\beta$-elimination reaction in which the leaving group is the intact hydroxyamino acid of the peptide core rather than the carbohydrate. Our studies show that under reasonably mild conditions OSM can be completely deglycosylated by this method. The mechanism of the reaction indicates that all mucin oligosaccharides containing peptide linked GalNAc residues with free hydroxyl groups at C3 and C4 will be similarly labile. By combining mild TFMSA treatment at $0^{o}$ with this "reverse $\beta$-elimination" reaction, it is expected that any mucin can be readily and completely deglycosylated. The time course and pH optimum of the reaction and the extent that these procedures may degrade the mucin peptide core will be discussed.

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156

VISCOSITY OF IONIZED COMPLEX CARBOHYDRATES AND POLYELECTROLYTES. Leon L. Yuan. Loyola University Dental School, Maywood, Illinois, 60153; Angela L. Yuan. Acadia Dental Association, Westmont, Illinois, 60559.

The popularity of the viscosity method is due to the ease with which experimental data can be obtained. However, the correlation of the experimental data to the physical parameters of the ionized complex carbohydrates and other polyelectrolytes has been difficult to obtain. Without this information, the theoretical studies of the viscosity of polyelectrolyte solutions became limited. Such limitation is not due to the simplicity of the method itself, but due to the lack of precise method of the data process.

In this article, we are reporting an unique method of processing the viscosity data by using the following two equations:

$$\eta_{sp}/c = [\eta]_{\infty}[1 + k/\sqrt{c}] \quad (1)$$

$$k = k_{o}[1 - e^{-g\sqrt{c}}] \quad (2)$$

A highly sulfated mucopolysaccharide, heparin, was used in our study. We have found the parameter $[\eta]_{\infty}$ represents the intrinsic viscosity of the well-shielded polyion and corresponds to the values of $[\eta]$ determined in concentrated salt solutions. During the acid hydrolysis study of heparin, we found that the parameter $k[\eta]_{\infty}$ is directly proportional to the number of titratable groups of the heparinic acid, as well as, the blood anticoagulant activity of the hydrolyzed heparin. In the study of the effect of dielectric constant (D) of the solvent on the viscosity of heparin, we found that parameter $[\eta]_{\infty}$ increases linearly with increasing the dielectric constant whereas the k values were independent of D. The viscosity of heparin is very much ionic strength dependent. We found parameter g is inversely proportional to the ionic strength.

## 157

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPIC DETERMINATION OF THE STRUCTURE OF THE CELL WALL REPEATING HEXASACCHARIDE FROM *STREPTOCOCCUS SANGUIS* H1.

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*Streptococcus sanguis* is a predominant species in the formation of dental plaque. NMR spectroscopy was used to determine the structure of the repeating hexasaccharide from the *S. sanguis* H1 carbohydrate antigen. This antigen acts as the receptor for the adhesin on *Capnocytophaga ochracea* ATCC 33596. Composition and methylation analyses showed the hexasaccharide to consist of terminal rhamnose/2-linked rhamnose/3-linked galactose/4-linked glucose = 1/1/3/1 with a galactosyl residue at the reducing end. GLC analysis of the trimethylsilyl (+/-)-2-butyl glycosides showed that the rhamnosyl and hexosyl residues were of the L and D configurations, respectively. The assignment of the proton resonances for each glycosyl residue was accomplished by taking 1-D $^{1}H$, $\{^{1}H,^{1}H\}$ COSY, and $\{^{1}H,^{1}H\}$ Hartmann-Hahn (HOHAHA) spectra. Using the proton assignment, the assignment of the carbon resonances was made from a $^{13}C$-detected $\{^{13}C,^{1}H\}$ one-bond shift correlation (HETCOR) spectrum. Three-bond couplings from the anomeric protons across the glycoside bonds to the carbons involved in these bonds on the adjacent residue were detected by recording a $\{^{1}H,^{13}C\}$ multiple-bond correlation (HMBC) spectrum. With this information and the proton and carbon assignments for each glycosyl residue, the complete glycosyl sequence of this hexasaccharide was determined to be as follows:

α-L-Rhap-(1→2)-α-L-Rhap-(1→3)-α-D-Galp-(1→3)-β-D-Galp-(1→4)-β-D-Glcp-(1→3)-α/β-D-Galp

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## 158

"LINK" PROTEINS OF HUMAN TRACHEOBRONCHIAL AND SALIVARY MUCINS. M.S. Reddy, M.J. Levine and P.C. Jones. Department of Oral Biology, School of Dental Medicine, SUNY at Buffalo, Buffalo, NY 14214.

"Link" proteins have been isolated from human and rat intestinal, pig gastric and human tracheobronchial (TBM) mucins. They are thought to serve as cross-linking peptides in the mucins via covalent linkages. Compared to mucins, link proteins are smaller in size, have higher amounts of Asx, Glx, Cys and may be glycosylated. In the present study, the "link" proteins from TBM and human salivary mucin (MG1) have been further characterized. TBM and MG1 were prepared by gel filtration on Sepharose CL-2B under dissociating conditions. Reductive methylation (RM) of TBM yielded two types of components: 1) mucin (90.8%; w/w) and 2) a pool (9.2%; w/w) rich in Asx+Glx (21.3%) and Cys (6.6%). The mucin component upon reduction of disulphide bonds (RA) yielded trace amounts of a fraction rich in Asx+Glx (22.3%) and Cys (6.5%). RM of MG1 also yielded two types of components: 1) mucin (73%; w/w) and 2) a pool (27.0%; w/w) rich in Asx+Glx (20%). The salivary mucin component upon RA yielded a pool (8.9%; w/w) rich in Asx+Glx (18.1%) and Cys (8.2%). The above experiments were then repeated in the reverse order. RA of TBM yielded two pools; mucin (85.7% w/w) and lower molecular weight fractions (14.3%; w/w) with Asx+Glx (20.0%) and Cys (6.5%). RM of RA-TBM mucin did not yield any additional fractions. RA of MG1 yielded mucin (94.6%; w/w) and a pool (5.4%; w/w) with Asx+Glx (19.6%) and Cys (2.8%). RM of RA-MG1 mucin yielded small amounts (~ 1%; w/w) of lower molecular weight fraction(s) with Asx+Glx (15.6%) and Cys (5.8%). These observations suggest that Asx,Glx,Cys enriched lower molecular fractions of TBM and MG1 may be covalently and/or non-covalently bound to mucin components. Therefore, some of the "link" proteins reported in the literature may or may not be disulphide-linked to their respective mucins.

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**159**

## CONFORMATION OF LEWIS BLOOD GROUP OLIGOSACCHARIDES FROM NOESY SIMULATIONS.

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Using the conditions of low temperature (5°C ) and solvent ($D_2O$, DMSO: $D_2O$) to modify the rotational correlation times and thus increase cross peak intensities, nuclear overhauser enhancements were measured for Lacto-N-difucohexaose (LND-1) and Lacto-N-fucopentaose (LNF-2). Two- dimensional 500 and 300 MHz NOESY experiments were done at mixing times of 250-900 ms and selective $T_1$ experiments were conducted under similar conditions to enable analysis of NOESY data at a single mixing time. A complete relaxation matrix method was used in the simulations, wherein experimental (direct and remote) NOE's were compared with theoretical intensities of computer-generated tetra- and tri-saccharide models obtained by systematic variation of glycosidic dihedral angles. The NOESY simulations, which include some small crosspeaks between protons on residues which are not directly connected by chemical bonds, showed a very narrow range of conformations in which experimental and theoretical NOE's agreed. Conformational energy calculations on similarly generated tri- and tetra-saccharide models indicated several minimum energy conformations, but only one corresponding to the NOE results.

We present a conformational model consistent with the NOESY and energy simulations in which the α-fucosyl residue linked to $Gal^3$ in LND-1 is folded close to the GlcNAc residue. In both LND-1 and LNF-2, the fucosyl residue linked to GlcNAc is close to the $Gal^3$ residue, as shown by remote NOE between $Fuc^4$ $H_5$ and $Gal^3$ $H_2$. The agreement of the simulated NOESY spectra for a single compact conformation with experimental data and with energy minima suggests that these two oligosaccharides in both $D_2O$ and in DMSO:$D_2O$ exist in a single conformation or a small group of closely related conformations.

**160**

## SYNTHESIS OF 4-NITROPHENYL O-β-D-GALACTOPYRANOSYL-(1→4)-O-(2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSYL)-(1→3)-β-D-GALACTOPYRANOSIDE AND A RELATED TRISACCHARIDE.

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For the past several years our group has been actively engaged in the synthesis of some oligosaccharides that are primarily intended for use in studies related to glycosidases and glycosyltransferases. Recently, we extended our efforts to encompass the synthesis of a variety of oligosaccharides that are amenable to further manipulation to produce artificial or synthetic antigens. Thus, in connection with the series of studies concerning both biochemical and immunological studies, we have synthesized two trisaccharides, 4-nitrophenyl O-β-D-galactopyranosyl-(1→4)-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→3)-β-D-galactopyranoside and methyl O-(2-O-methyl-β-D-galactopyranosyl)-(1→4)-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→3)-β-D-galactopyranoside. Synthetic schemes for these two compounds will be presented.

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## 161

SYNTHESIS OF BENZYL 2'-O-METHYL-LACTO-N-BIOSE I AND ITS HIGHER SACCHARIDES - POTENTIAL SUBSTRATES FOR α-L-(1→4)-FUCOSYLTRANSFERASE.

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At present, greater attention is being focussed upon the enzymatic basis for the aberrant accumulation of tumor-associated antigens in tumor cells so that a crucial enzyme responsible for the synthesis of that antigen may be identified and used as a diagnostic marker for cancer. One such enzyme involved in the synthesis of CA 19-9 antigen is α-L-(1→4)-fucosyltransferase. Therefore, the availability of compounds capable of acting as acceptors for a single enzyme, even in the presence of other, related enzymes, would be of particular importance. In this regard, we have recently synthesized benzyl 2-acetamido-2-deoxy-3-O-(2-O-methyl-β-D-galacto-pyranosyl)-β-D-glucopyranoside (benzyl 2'-O-methyl-lacto-N-biose I) and it has been found to be an efficient acceptor for α-L-(1→4)-fucosyltransferase. In addition, we have also synthesized a trisaccharide, MeO-2Galβ1→3GlcNAcβ1→3Galβ1→OMe, and a tetrasaccharide, MeO-2Galβ1→3GlcNAcβ1→3Galβ1→4Glc incorporating 2'-O-methyl-lacto-N-biose I as a terminal unit. Strategies towards the chemical synthesis of these different acceptors will be presented.

This investigation was supported by PHS Grant No. CA-35329 awarded by the National Cancer Institute, DHHS, and in part by Grant No. CH 419 awarded by the American Cancer Society.

## 162

A FACILE SYNTHESIS OF FUCOSYLATED OLIGOSACCHARIDES.

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A variety of glycoconjugates with carbohydrate sequences containing α-L-fucose have been characterized as tumor-associated antigens. Similar structures have also been identified in normal tissues as well. For this reason we have embarked on a program for the synthesis of some fucosylated oligosaccharide fragments that occur as part of glycoconjugates. For the synthesis of α-L-linked fucose oligosaccharides methyl 2,3,4-tri-O-acetyl-1-thio-β-L-fucopyranoside, obtained from L-fucose tetra acetate, trimethylsilyl triflate and (methylthio)trimethylsilane, was converted into methyl 2,3,4-tri-O-benzyl-1-thio-β-L-fucopyranoside which was utilized as a glycosylating agent and condensed with suitably protected acceptors under catalysis by cupric bromide-tetrabutyl ammonium bromide. The synthesis of β-D-Gal-(1→3)-[α-L-Fuc-(1→4)]-β-D-GlcNAc-(1→3)-β-D-Galβ1→OMe, α-L-Fuc-(1→3)-β-D-Galβ1→OMe and α-L-Fuc-(1→2)-α-L-Fucα1→OMe have been accomplished using methyl 2,3,4-tri-O-benzyl-1-thio-β-L-fucopyranoside as the glycosyl donor.

This investigation was supported by grant #CH 419 awarded by the American Cancer Society and in part by Grant #CA 35329 awarded by the National Cancer Institute, DHHS.

**163**

DETERMINATION OF THE COMPLETE STRUCTURE OF CELL WALL POLYSACCHARIDE FROM *STREPTOCOCCUS SANGUIS* ATCC 10557 BY HIGH FIELD NMR SPECTROSCOPY

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The cell wall polysaccharides of certain oral streptococci such as *Streptococcus sanguis* strains 34, J22 and ATCC 10557, although immunologically different, act as receptors for the fimbrial lectin of *Actinomyces viscosus* T14V. We report the complete covalent structure of the polysaccharide from *S. sanguis* ATCC 10557 which is composed of a hexasaccharide repeating subunit linked by phosphodiester bonds. The repeating subunit, which contains $\alpha$-Galactose, $\beta$-Galactose, $\beta$-Rhamnose $\beta$-Glucose and $\alpha$-GalNAc all in the pyranosidic form and $\beta$-Galactofuranose is compared with structures of the polysaccharides from *S. sanguis* strains 34 and J22. The polysaccharide from *S. sanguis* ATCC 10557 was also shown to have O-acetylation in two hydroxyl positions on about one-third of the repeating subunit.

The structure has been determined almost exclusively by high resolution nuclear magnetic resonance methods. The $^{1}H$ and $^{13}C$ NMR spectra of the polysaccharide have been completely assigned. Proton spectra were assigned by DQF-COSY, 2D-HOHAHA and TQF-COSY, and stereochemistry of the pyranosides was assigned from coupling constant values determined from phase-sensitive COSY spectra. The $^{13}C$ spectra were assigned by proton- detected multiple quantum correlation (HMQC) spectra and the assignments were confirmed by proton-detected multiple bond correlation (HMBC) spectra. The positions of the glycosidic linkages as well as O-acetylation sites were assigned by detecting three-bond $^{1}H$-$^{13}C$ correlation across the glycosidic linkage in the HMBC spectra. The positions of the phosphodiester linkages were determined by splitting observed in the $^{13}C$ resonance due to $^{31}P$ coupling and also by proton-detected $^{31}P$ correlation spectroscopy.

**164**

STRUCTURAL STUDIES ON MUCINS FROM CYSTIC FIBROSIS PATIENTS AND CONTROLS. Rekha Gupta and Neil Jentoft. Case Western Reserve Univ. Cleveland, OH 44106

The size, composition, and structure of tracheobronchial mucins from both cystic fibrosis (CF) patients and tracheotomized control patients were investigated. Mucins were purified by extracting secretions with 6 M guanidine hydrochloride containing protease inhibitors followed by treatment with DNAse and hyaluronidase. An initial gel filtration step using Sephacryl S-1000 separated intact mucins from proteolytically degraded mucin fragments. The high molecular weight fraction was further purified by density gradient centrifugation followed by rechromatography on Sephacryl S-1000.

The most notable difference between the CF and control samples was in the size distribution of the mucins. Control samples consisted almost entirely of high molecular weight mucins (MW > $2x10^6$) while CF samples contained some high molecular weight mucins but largely consisted of smaller mucin fragments. This is probably due to proteolytic degradation in the CF respiratory tract as suggested by Rose et al. (Pediat. Res. 22 545, 1987). No difference was seen in the lipid content of purified CF and control mucins. Comparisons of carbohydrate compositions between control and CF mucins were complicated by high levels of individual variability but no CF-specific differences were seen in the limited number of samples tested.

The structure of the high molecular weight fraction of CF and control tracheobronchial mucins appears to be similar. Reduction and alkylation releases subunits of $2x10^6$ Da together with a 65 kDa link protein while subsequent treatment of the subunits with trypsin, endoproteinase K, or human neutrophil elastase releases glycosylated domains of ca. 300 kDa. Thus the structure of tracheobronchial mucins appears to be similar to that described for cervical (Carlstedt et al., Essays in Biochemistry 20 40, 1985) and salivary (Gupta and Jentoft, Biochemistry, In Press) mucins.

*(Supported by grants from the Cystic Fibrosis Foundation and by AM-27651)*

## 165

### THE CARBOHYDRATE STRUCTURES OF *TRYPANOSOMA BRUCEI BRUCEI* MITAT 1.6 VARIANT SURFACE GLYCOPROTEIN

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*Trypanosoma brucei brucei* is a causative agent of African trypanosomiasis. The parasite lives in the body fluids of its mammalian host, protecting itself by the sequential expression of antigenically distinct glycoproteins over its entire cell surface. These variant surface glycoproteins (VSGs) possess *N*-linked oligosaccharide chains whilst being anchored in the cell membrane by a glycosylphosphatidylinositol (GPI) moiety. We have studied both the *N*-linked carbohydrates and the GPI anchor of such a VSG, namely that of *T.b.brucei* MITat 1.6.

*N*-Glycopeptides were released enzymatically and, after purification, found to be homogeneous in their peptide portion. The latter was shown to be Asn-Ala-Thr by the combination of amino acid analysis, 1-D $^1$H-NMR spectroscopy and 2-D $^1$H,$^1$H-COSY spectroscopy. The oligosaccharide portion of the *N*-glycopeptides consisted of GlcNAc and Man in the ratio of 2:8, as determined by glycosyl composition analysis. The NMR spectra showed the characteristic structural-reporter-group signals for high-mannose structures. The glycopeptides were found to be heterogeneous with respect to their carbohydrate structures with structures ranging in size from $Man_7GlcNAc_2$ to $Man_9GlcNAc_2$.

The structure of the *C*-terminal glycan moiety of the GPI anchor was studied by $^1$H- and $^{31}$P-NMR spectroscopy and chemically under varying conditions of acid hydrolysis and enzyme digestions. The glycan consisted of Asp, ethanolamine, non-*N*-acetylated GlcN, Man, Gal and phosphate. 1-D $^1$H-NMR and 2-D $^1$H,$^1$H-COSY NMR spectroscopy at 500 MHz showed that the structure of the glycan is similar to that reported for MITat 1.4 [1], but significantly different to that reported for MITat 1.6 [2]. The core of MITat 1.6 GPI *C*-terminal glycan is identical to that of MITat 1.4 with a variable number of α-Gal residues attached.

These data are relevant to the study of African trypanosomiasis in the light of recent evidence for the involvement of high-mannose oligosaccharides in immunosuppression and the knowledge that immunosuppression is characteristic for African trypanosomiasis, as well as with the idea of the GPI anchor being a potential target for chemotherapy.

[1] MAJ Ferguson, SW Homans, RA Dwek & TW Rademacher (1988) *Science* 239: 753-759
[2] B Schmitz, RA Klein, IA Duncan, H Egge, J Gunawan, J Peter-Katalinic, U Dabrowski & J Dabrowski (1987) *Biochem Biophys Res Commun* 146: 1055-1063
[Research supported by NIH Grant HL-38213]

## 166

### LOW ENERGY COLLISIONAL ACTIVATION TANDEM MASS SPECTROMETRY CAN DISTINGUISH AMONG LINKAGE POSITIONS IN UNDERIVATIZED OR METHYLATED SMALL OLIGOSACCHARIDES.

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Sets of neutral heterooligosaccharides containing fucose, galactose and N-acetylglucosamine were synthesized which differed only in the position of linkage of the terminal monosaccharide to the penultimate GlcNAc. Low energy collision activation after fast atom bombardment ionization (FAB-MS-CID-MS) showed that patterns of collision of the molecular ion and glycosidic cleavage fragment ions were unique for the linkage position when the spectra were recorded at conditions where the relative intensity of the parent ion for the 1-4 linkage was 20% (Laine, et al. JACS 110: 6931 (1988)). These experiments were correlated with molecular modeled rotational freedom of the linkage in question. Subsequent experiments have shown that more sensitive discrimination may be seen by preserving a greater percentage of the molecular ion, and that discrimination can be seen among CID spectra of the alkali series cationized species in a stability set K>Na>Li. Permethylated oligosaccharides were prepared and subjected to FAB-MS-CID-MS. A unique ion appears for the 3 linkage in both terminal fucose and terminal galactose series, and the daughter ion patterns are different among the different linkage positions. (Supported in part by grants to RAL from the NIH).

# Glycoproteins

## 201

T200 GLYCOPROTEIN: POLY-*N*-ACETYLLACTOSAMINE IS ASSOCIATED WITH ALTERNATE EXON USE IN T200 FROM LYMPHOKINE ACTIVATED NATURAL KILLER CELLS. H. Beittenmiller, H.-L. Chang and W.J. Esselman. Department of Microbiology, Michigan State University, E. Lansing, MI 48824-1101.

Previous work has suggested a functional role for poly-*N*-acetyllactosamine expression on T200 glycoprotein in the formation of conjugates between natural killer cells and tumor-target cells (J. Immunol. 140:2821,1988). T200 (also called CD45, LCA, B220) exhibits cell lineage specific heterogeneity due to three alternate exon-coded sequences and to glycosylation. In this report we describe the analysis of the glycosylation of different isoforms of T200 isolated from lymphokine activated natural killer cells (LANK), B cells (70/Z) and T cells (BW5147). The cells were metabolically labeled with [6-$^3$H]Gal followed by isolation of different cellular $M_r$ isoforms of T200 by gel purification. The T200 was digested by protease and the resultant glycopeptides were subjected to analysis using lectins specific for polylactosamine (tomato lectin, TL; and potato lectin, PL). The three cell types all expressed polylactosamine, but T200 associated polylactosamine was found only from those cells in which T200 contained one or more alternate exon-coded sequences. LANK cell T200 expressed particularly high levels of polylactosamine (up to 40% of LANK [$^3$H]Gal-glycopeptides bound to TL). Endo-$\beta$-galactosidase treatment of TL-bound [$^3$H]Gal-glycopeptides caused the release of radioactivity predominantly in the form of trisaccharides and disaccharides suggesting the presence of branched polylactosamine structures. The TL-bound [$^3$H]-glycopeptides from LANK T200 were found to be excluded from BioGel P-6 and P-10 columns. Treatment of these large polylactosamine-containing glycopeptides with alkaline/borohydride resulted in the release of all of the [$^3$H]Gal label. We conclude that protease digestion of T200 results in both small glycopeptides as well as large protease-resistant glycopeptides formed by the alternate exon-coded domains which are rich in *O*-glycans and which contain essentially all of the T200 polylactosamine. The expression of polylactosamine on T200 was associated with alternate exon use and the regulation of this use may control the function of T200 in the NK tumor-target adhesion process.

## 202

**CHARACTERISTICS OF A SULFATED HUMAN PANCREATIC ADENOCARCINOMA MUCIN GLYCOPROTEIN.**

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**An experimental approach utilizing cell culture in conjunction with monoclonal antibody technology has led to the preparation of a monoclonal antibody (DU-PAN 2) that is currently used in diagnosis and in monitoring the therapy of pancreatic adenocarcinomas. The antigen recognized by the antibody is a developmentally regulated, anionic, mucin glycoprotein, with the anionic properties attributable to sialic acid and sulfated saccharide residues. The mucin is isolated by ammonium sulfate fractionation followed by molecular sieve and density gradient centrifugation. No contaminating proteins or glycoproteins were detected by standard electrophoresis analyses, in reducing-denaturant buffers, on 7% polyacrylamide and 1% agarose gels. Furthermore, no unreactive DU-PAN 2 mucin species have been detected by affinity chromatography on an antibody-Sepharose 4B bed. The purified mucin contains sialic acid, sulfate, galactose, fucose, acetylglucosamine, acetylgalactosamine, and no detectable mannose. The results of enzymatic and chemical analyses show that the oligosaccharide chains contain 9 units that are arranged in a manner closely related to the central biantennary composite structure of ovarian cyst mucin proposed by Llyod and Kabat in 1968, but with the following features that distinguish the pancreatic mucin from ovarian cyst and other human mucins: (1) chromatography of the reductive β-elimination product on DEAE yielded only anionic oligosaccharides, which contrasts to the prominence of neutral oligosaccharides in human ovarian cyst and tracheobronchial mucins. (2) all of the oligosaccharide chains are sulfated , and the sulfate is esterified to the six position of one of the acetylglucosaminyl residues. Unlike human tracheobronchial mucin, no o-sulfated galactosyl residues were detected. (3) the terminal galactosyl residues on each branch are substituted on the three position with sialic acid, which is the position of substitution by the α-linked acetylgalactosaminyl or galactosyl residues in the A and B blood group substances respectively.**

203

## ALTERED GLYCOSYLATION IN HEPATOCELLULAR CARCINOMA CELLS.

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Changes in membrane glycosylation in malignant cells may be reflected in functional alterations of tumor cells. These changes may occur either in membrane glycoproteins or glycolipids. Such structurally altered glycoconjugates may have a potential use as tumor markers when recognized by monoclonal antibodies.

To test glycosylation in hepatoma cells, we labeled human hepatoma cell lines BEL–7402, QGY–7703 and SMMC–7721 with D–[2–$^3$H]–mannose and D–[$^3$H]–glucosamine. Autoradiography of labelled cell glycoproteins separated by SDS–PAGE showed that Mr 137 KD glycoprotein(s) exhibited increased labeling compared to Chang Liver cells, a normal human liver cell line. Chang Liver cells showed intense labeling in Mr 75 KD glycoprotein(s) while all three hepatoma cell lines exhibited retarded migration of the Mr 75 KD glycoprotein(s). L–PHA blotting analysis revealed that the hepatoma cell Mr 137 KD glycoprotein(s) had a higher binding activity, indicating increased –GlcNAc ß 1→6 Man α 1→6 Man ß– branching in N–linked oligosaccharides.

Changes in glycosylation of membrane glycoconjugates must be mediated by alterations in glycosyltransferase activity. In previous studies, we have quantitated the expression of disialolactosylceramide (GD3) and CMP–NeuAc:GM3 sialyltransferase activity in human and rat hepatomas. The activity of CMP–NeuAc: GM3 sialyltransferase was enhanced 0.5–3 fold over that of normal liver tissues. The activity of this enzyme also increased in relation to the progress of tumorigenesis in diethylnitrosamine induced rat hepatoma. Consequently, we detected an accumulation of GD3 in all the hepatoma samples tested.

The activity of CMP–NeuAc: GM3 sialyltransferase was also found to have increased in NIH 3T3 cells transfected with human tumor DNAs. Whether these changes in glycosyltransferase properties can be associated with oncogene products continues to be an intriguing possibility.

204

## THROMBOSPONDIN PROMOTES ARTICULAR CHONDROCYTE ATTACHMENT BUT NOT SPREADING.

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Thrombospondin, a high molecular weight glycoprotein synthesized and secreted by various cells in culture, is similar in structure and function to the members of the class of adhesive proteins. Amino acid sequencing revealed the presence of the cell binding sequence arg-gly-asp (RGD) in thrombospondin. Consistent with this finding, thrombospondin has been shown to support the attachment of several normal and transformed cell types. In the present study we examined the ability of thrombospondin to mediate the attachment of articular chondrocytes. Cell attachment assays were performed in microwells using a spectrophotometric assay based on the ability of chondrocytes to reduce a tetrazolium salt, MTT, to form a blue formazan product. When added to thrombospondin coated microwells, chondrocytes readily attached but failed to spread in a three hour incubation. By comparison, chondrocytes failed to attach to wells coated with bovine serum albumin. The effect of thrombospondin on attachment is specific since preincubation of the thrombospondin coated wells with monoclonal antibody to thrombospondin essentially abolished chondrocyte attachment. When chondrocytes were added to thrombospondin coated wells in the presence of the synthetic peptide RGD, attachment was inhibited. No inhibition of chondrocyte attachment was observed when the synthetic peptide RGES was present. Thus, thrombospondin appears to mediate chondrocyte attachment through its RGD sequence. Finally, chondrocyte attachment requires divalent cations since chondrocyte attachment was completely inhibited if the thrombospondin coated wells were preincubated with EDTA.

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## 205

A RAT OSTEOBLASTIC CELL LINE (UMR 106-01) SYNTHESIZES A SULFATED FORM OF A BONE SIALOPROTEIN. R. J. Midura, D. J. McQuillan, and V. C. Hascall. Bone Research Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20892

A rat osteoblastic cell line (UMR 106-01) synthesizes a sulfated glycoprotein that co-purifies with proteoglycans on ion exchange chromatography. When cultures are labeled with [$^{35}$S]sulfate for 24 hours, ~20% of the total incorporated radioactivity is present in this glycoprotein with ~1/3 in the cell layer and ~2/3 in the medium. The macromolecule was purified by Q-Sepharose and Superose 6 chromatographic steps. The amino terminal sequence through 10 residues was determined and shown to be identical with rat bone sialoprotein (BSP) (1), which has not been shown previously to be sulfated. BSP was isolated from cultures labeled for 24 hours with [$^{35}$S]sulfate and [$^{3}$H]glucosamine in the presence or absence of tunicamycin. Alkaline borohydride digests of BSP were eluted on Bio-Gel P-10 (2). About 70% of the $^{35}$S eluted near the total volume with no $^{3}$H, and was shown to be derived from tyrosine sulfate by established procedures (3). About 20% of the $^{35}$S and ~45% of the $^{3}$H eluted early in a broad peak where N-linked glycopeptides elute (2), and this peak was virtually absent in BSP from tunicamycin-treated cultures. The remaining ~10% of the $^{35}$S and ~45% of the $^{3}$H eluted in two peaks where O-linked hexa- and tetrasaccharides elute (2). The combined O-linked oligosaccharide fraction was applied to a CarboPac PA1 anion exchange column and eluted with a sodium acetate gradient (0-0.4 M) in 0.1 M NaOH. Three major and four minor $^{3}$H peaks (~80% of the total O-linked $^{3}$H) eluted early in the gradient. Three (~10% of the total O-linked $^{3}$H) eluted later with two containing ~90% of the total O-linked $^{35}$S. These latter two sulfated oligosaccharides eluted on Bio-Gel P-10 as a tetra- and a hexasaccharide. All of the O-linked oligosaccharides contained galactosaminitol in hexosamine analyses, confirming their O-linked structures. Thus the UMR 106-01 osteogenic cells synthesize a sialoprotein characteristic of bone tissue which contains sulfated tyrosine and lesser amounts of sulfated N- and O-linked oligosaccharides.

1. Oldberg, Å., Franzén, A., and Heinegård, D. (1988) J. Biol. Chem. **263**, 19430.
2. Lohmander, L. S., De Luca, S., Nilsson, B., Hascall, V. C., Caputo, C. B., Kimura, J. H., and Heinegård, D. (1980) J. Biol. Chem. **255**, 6084.
3. Huttner, W. B. (1984) Methods Enzymol. **107**, 200.

## 206

A PUTATIVE 'LINK' COMPONENT IS PRESENT IN RESPIRATORY MUCIN OF PATIENTS WITH CYSTIC FIBROSIS. U. Sajjan, J.F. Forstner, Hospital for Sick Children, Toronto, Ontario Canada.

Earlier we have demonstrated that a 118 kDa glycopeptide is present as an integral component of both control and CF intestinal mucins (J. Biol. Chem. 260, 7955, 1985; Biochem J. 261, 637, 1989). A similar component of approx 150 kDa was also present in human salivary mucin. In the present study we report the presence of a 'link' component in tracheobronchial (sputum) mucin isolated from patients with cystic fibrosis. Sputum was collected in PBS containing 6M GdnHCl, 2mM PMSF and 0.02% $NaN_3$, and the mucin purified by the method of Carlstedt et al (Biochem J 213, 427, 1983). The mucin had an amino acid and carbohydrate profile typical of other reported respiratory mucins, and was free of fibronectin. On SDS-PAGE, the mucin did not show any small mol wt contaminants. Upon reduction with β-mercaptoethanol, a broad band with a mid-point position of mol wt approx. 138 kDa was released. This component contained 3.3 mol % mannose as well as other typical mucin carbohydrates. Like intestinal mucins, the sputum mucin bound type 1 piliated *E. coli* O157:H7 strain CL-49. The 138 kDa glycopeptide, like the intestinal 118 kDa 'link' glycopeptide, was an effective inhibitor of type 1-piliated *E. coli* CL-49 binding to mucin. These findings indicate that the sputum mucin, like intestinal mucin, possesses a 'link' glycopeptide containing high-mannose N-linked oligosaccharide chains.

Financial support received from the Canadian Cystic Fibrosis Foundation.

THE 'LINK' GLYCOPEPTIDE OF INTESTINAL MUCIN BINDS TO TYPE 1 PILI OF *E. COLI* O157:H7, STRAIN CL-49 BY MANNOSYL-CONTAINING RECEPTORS AND STRONG HYDROPHOBIC INTERACTIONS. U. Sajjan, G. Forstner, J. Forstner Hospital for Sick Children and University of Toronto, Toronto, Ontario, Canada.

Following reduction of disulfide bonds, highly purified gastrointestinal mucins of several species depolymerize and release a component of approx Mr of 118 kDa. Immunolocalization studies have confirmed that this putative 'link' glycopeptide is present in goblet cell granules and secreted mucin of rat intestine (Biochem J 243, 631, 1987). In the present study purified mucins were shown to bind to type 1 piliated (mannose-sensitive) *E. coli* O157:H7, strain CL-49, but not to other *E. coli*. type 1-pili. The mucin receptor sites for *E. coli* CL-49 were identified by hapten inhibition and direct binding experiments, using $^{3}$H-labelled *E. coli* CL-49 and a microtiter binding assay. Bacterial binding was specific for the 118 kDa 'link' glycopeptide of mucin, was inhibited by D-mannose and α-linked mannosyl derivatives, particularly man α 1,3 β 1,4 GlcNAc; and was abolished by an antibody to type 1 pili of an enteropathogenic *E. coli* RDEC-1. Preincubation of the 118 kDa glycopeptide with jack bean α-mannosidase or Endo H inhibited bacterial binding. Thus piliated *E. coli* CL-49 have adhesion specificity typical of type 1 pili, and the 'link' component of mucin contains high mannose N-linked oligosaccharides. Compositional analyses of the isolated 'link' component confirmed the presence of mannose (10 mol %).

Since type 1 pili of other *E. coli* strains did not bind to mucin, some features of strain CL-49 must be unique. Comparisons of several type 1-piliated *E. coli* in 3 assays of hydrophobicity showed that the pili of CL-49 were much more hydrophobic than the others. Further, binding of strain CL-49 to mucin was inhibited by p-nitrophenol and tetramethylurea. Thus mucin 'link' glycopeptide contains mannosyl receptor sites for type 1 pili, but the stable binding of E. coli CL-49 appears to require, in addition, uniquely strong hydrophobic interactions between the pili and the mucin 'link' component.

# Glycosyltransferases

**208**

PURIFICATION AND CHARACTERIZATION OF FUCOSYLTRANSFERASES FROM BOVINE SPLEEN. J.W. Hawes, M. Basu and S. Basu, Dept. of Chem. and Biochemistry, Biochem.Biophys. and Mol.Biol.Prog., Univ. of Notre Dame, Notre Dame,IN

The lacto-N-biose core (Gal β1-3GlcNAc-; Lc-) and N-acetyllactosamine core (Gal β1-4GlcNAc-; nLc-)-containing glycoconjugates comprise the ABO and Lewis blood group systems of eukaryotic cell surfaces. We previously reported the characterization of fucosyltransferases from bovine spleen (BS) that catalyze the transfer of fucose from GDP-fucose to the C-2 position of the terminal galactose moiety of nLcOse4Cer (FucT-2; Basu,S. et al. (1975) J.Biol.Chem., 250, 2956-2962) and to the C-3 position of the penultimate N-acetylglucosamine unit of nLcOse4Cer (FucT-3; Basu, S. et al.(1988) Biochimie, 70, 1551-1563) to form blood groups H and $Le^x$, respectively. The detergent- (TDC) solubilized Golgi-rich membranes from BS revealed at least two fucosyltransferase activities as well as the presence of a highly active endogenous fucosyl acceptor. The endogenous glycoprotein-active acceptor was removed by a RCA-I lectin affinity column. Further purification of FucT-2 (GDP-Fuc: Galβ1-4GlcNAc-R α1-2FucT) has been achieved by DEAE-CL6B ion-exchange and specific affinity chromatography (GDP-agarose). The purified FucT-2 was active with nLcOse5Cer as well as the exogenous glycoprotein substrates (SA, α-1-acid glycoprotein and ovalbumin). The Vmax value with N-acetyllactosamine (Galβ1-4GlcNAc) was 4-fold higher than with lacto-N-biose (Gal 1-3GlcNAc). Among all the acceptors tested, nLcOse5Cer had the lowest Km value(33 μM). Using theHVE-RDAC assay (Basu, M. et al.(1987) Methods Enzymol., 138, 575-607) fucose incorporation from GDP-$^{14}$C-fucose in glycoprotein has been studied in the presence of different potential GSL acceptors. GgOse4Cer (Galβ1-3GalNAcβ1-4Galβ1-4GlcCer) does not accept fucose, but it is a good inhibitor. In addition to the FucT-2 activity, whether FucT-2' activity (GDP-Fuc: Galβ-1-3GlcNAc-R α1-2FucT) is due to the presence of a separate gene product or due to the dual specificity of a same enzyme is not known and is under study. (Supported by NIH Grants NS-18005 , C14764 and CA-33751)

**209**

*IN VITRO* MEASUREMENT OF β-GALACTOSYLTRANSFERASE ACTIVITY IN WHOLE CELL EXTRACTS WITH AN ENZYME-LINKED IMMUNOSORBENT ASSAY SYSTEM. Cheryl L.M. Stults and Bruce A. Macher.
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Glycosyltransferases are of primary importance in the biosynthetic pathway associated with the expression of glycosphingolipid antigens on cell surfaces. Therefore, an assay was developed in which glycosyltransferase activity could be conveniently measured *in vitro*. After a preparation containing the enzyme reacts with an immobilized glycosphingolipid substrate, product identification and quantification is accomplished with an enzyme-linked immunosorbent assay (ELISA), which utilizes monoclonal antibodies and an avidin-biotin alkaline phosphatase complex for detection with p-nitrophenylphosphate. This assay was used with detergent extracts of LEC 11 cells (a mutant of the chinese hamster ovary cell line, W5), which are known to express a complex array of carbohydrate structures. To measure UDP-Gal:N-acetylglucosamine β(1,4)-galactosyltransferase activity, a glycosphingolipid substrate, lactotriglycosylceramide ($GL_3$), was first immobilized in microtiter wells. A reaction mixture (containing UDP-Gal, buffer, and $MnCl_2$) and a detergent solubilized preparation of the enzyme (from whole cells) were then added to the well. After removal of the enzyme reaction mixture, the product, neolactotetraglycosylceramide, was identified with the monoclonal antibody, 1B2, which specifically binds to the Galβ1-4GlcNAc epitope. The enzyme activity in the preparations was found to be similar to that obtained by conventional radioactive assay methods. Optimal reaction conditions were determined by varying each of the following: pH, time, $GL_3$, UDP-galactose, and $MnCl_2$. The β-galactosyltransferase found in LEC 11 cell detergent extracts exhibited an absolute requirement for the nucleotide sugar and $MnCl_2$. The activity of the enzyme was also strictly dependent on the presence of exogenous glycolipid acceptor. The LEC 11 cells have provided a useful model to determine reaction conditions that can be used to screen other cell lines for β-galactosyltransferase activity. The assay procedure described here should also be applicable to measurement of other glycosyltransferase activities in whole cell extracts. These studies were supported in part by NIH grant #CA32826.

## 210

MECHANISM OF RELEASE OF Galβ1-4GlcNAcα2-6 SIALYLTRANSFERASE FROM GOLGI MEMBRANES. G. Lammers and J.C. Jamieson. Department of Chemistry, University of Manitoba, Winnipeg, Canada, R3T 2N2.

Inflammation in the rat, mouse and guinea pig results in the release of Golgi membrane-bound Galβ1-4GlcNAcα2-6 sialyltransferase (α2-6 ST) into the serum (Kaplan et al.,1983, J. Biol. Chem. 258:11505-11509; Lammers and Jamieson, 1986, Comp. Biochem. Physiol. 84:181-187; Lammers and Jamieson, 1988, Biochem. J. 256:623-631). We have studied the mechanism of release of α2-6 ST from the Golgi membrane in all three species. Golgi membranes were disrupted by sonication followed by incubation at reduced pH and release of α2-6 ST was monitored. Approximately 70% of the sialyltransferase was released at reduced pH (pH 5.6 for rat, pH 4.6 for mouse, and pH 5.2 for guinea pig). The release of α2-6 ST from liver slices was inhibited in the presence of the lysosomotropic agents $NH_4Cl$, chloroquine and methylamine which confirmed that sialyltransferase release from Golgi membranes is a pH-dependent phenomenon. Kinetic studies revealed that the released portions of the rat, mouse and guinea pig enzymes exhibited identical affinities for the substrates CMP-NeuAc and asialo-$\alpha_1$-acid glycoprotein. The rat and mouse Golgi membrane-bound sialyltransferases had $M_r$ of approximately 49,000, but the guinea pig enzyme had $M_r$ of about 42,000. The released α2-6 ST from rat Golgi membranes had $M_r$ of about 42,000, whereas those released from mouse and guinea pig Golgi membranes had $M_r$ of approximately 38,000. Studies with proteinase inhibitors showed that only pepstatin A, a potent inhibitor of cathepsin D-like proteinases, acted as an inhibitor of α2-6 ST release from Golgi membranes under the experimental conditions used. Release of rat liver α2-6 ST was also inhibited by preincubation with antiserum raised against rat liver lysosomal cathepsin D. The cathepsin D-like activity that released sialyltransferase was determined to be present at the luminal side of the Golgi membrane and could be removed by washing with mannose-6-phosphate. The conclusion is that the catalytic portion of α2-6 ST may be released from its membrane anchor by lysosomal cathepsin D. This proteolytic event is enhanced during the acute phase response which explains why the α2-6 ST is an acute phase reactant. Supported by the N.S.E.R.C. Canada.

## 211

BIOSYNTHESIS OF O-GLYCANS: USE OF SYNTHETIC GLYCOPEPTIDES AS ENZYME SUBSTRATES. I.Brockhausen and *H.Paulsen. Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada, and *Institute for Organic Chemistry, University of Hamburg, Hamburg, FRG

Many glycoproteins carry oligosaccharides with structures characteristic of the individual glycosylation sites. The peptide moiety therefore appears to influence the biosynthetic processing of oligosaccharides. We are interested in examining how the peptide sequence, length and composition, the presence of proline, as well as the presence of multiple glycosylation sites affect the biosynthetic pathways to the common mucin core structures. Series of glycopeptides containing one to ten amino acids were synthesized with Galβ1-3GalNAcα- or one or more GalNAcα- units linked to serine or threonine (Paulsen et al., 1988). These glycopeptides are tested as substrates in assays of the following enzymes from rat, pig, and human colonic, and pig gastric mucosa: 1. UDP-Gal:GalNAcα-R β3 Gal-transferase, synthesizing core 1 (Galβ3GalNAcα-); 2. UDP-GlcNAc:Galβ3GalNAcα-R β6 GlcNAc-transferase synthesizing core 2 (GlcNAcβ6 [Galβ3] GalNAcα-); 3. UDP-GlcNAc:GalNAcα-R β3 GlcNAc-transferase synthesizing core 3 (GlcNAcβ3GalNAcα-); and UDP-GlcNAc: GlcNAcβ3GalNAcα-R β6 GlcNAc-transferase synthesizing core 4 (GlcNAcβ6 [GlcNAcβ3] GalNAcα-). To obtain accurate estimates of enzyme activities assay components are separated by HPLC on silica based C18 columns with acetonitrile/water mixtures (0/100 to 20/100) as the mobile phase. Glycopeptide substrates and enzyme products elute according to their hydrophobicity. ref.: H.Paulsen, K.Adermann, G.Merz, M.Schultz und U.Weichert. (1988) Starch 40:465-472.

212

FORMATION OF AN α-MANNOSYL-N-ACETYLGLUCOSAMINE LINKAGE BY PORCINE LIVER MICROSOMES. John W. Jensen and John S. Schutzbach, Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294

A number of proteins have been shown to be attached to the cell surface by glycosyl-phosphatidylinositol (GPI) anchors and this glycolipid is known to contain a novel α-mannosyl-glucosamine linkage. We have investigated the formation of α-mannosyl-hexosamine disaccharides using the free sugars as mannosyl acceptors. Porcine liver microsomes catalyzed mannosyltransfer from GDP-[$^{14}$C]Mannose to free D-N-acetylglucosamine resulting in the synthesis of an α-mannosyl-N-acetylglucosamine linkage. Mannosyltransfer to N-acetylglucosamine was linear with time for at least 4 hours (0.6 mg protein) and proportional to enzyme concentration up to 1.2 mg protein. The reaction required divalent cation with $Mn^{++}$ providing maximal stimulation. The apparent $K_m$ for GDP-Mannose was 10 μM and the $K_m$ for the N-acetylglucosamine acceptor was 0.27 M. Formation of the mannosyl-N-acetylglucosamine disaccharide was inhibited by the presence of amphomycin suggesting that dolichol-P-mannose was the mannosyl donor. Under the same conditions, D-glucosamine was not an acceptor for mannosyl transfer suggesting the possibility that during GPI synthesis, the N-acetylglucosamine residue is N-deacetylated following mannosyl transfer. Work by others, however, suggests that the N-acetylglucosamine residue linked to phosphatidyl-inositol is N-deacetylated prior to the transfer of the initial mannose residue (Doering, T. L. et. al. (1989) J. Biol. Chem. 264, 11168-11173). Resolution of these differences will require purification of the mannosyltransferase and further structural characterization of the disaccharide product. (Supported by NIH grants CA16777 and GM38643)

213

**UDP-GALACTOSE: GLOBOSIDE GALACTOSYL TRANSFERASE IN THE MURINE KIDNEY.** **Koul, O., Prada-Maluf M., and McCluer, R.H. Biochemistry Dept. E.K. Shriver Center, Waltham, MA and Dept. Neurology Harvard Medical School, Boston, MA**

**Stage specific embryonic antigen-3 (SSEA-3) is a globo-series cell surface antigen on human teratocarcinoma cells, murine embryonic and adult DBA/2 kidney cells. The biosynthesis of this glycolipid antigen from its precursor, globoside, has not been studied in the kidney. Here we report the presence and characterization of the biosynthetic enzyme UDP-galactose: globoside galactosyl transferase in microsomes from kidneys of C57BL/6 (which contain SSEA-1 but little SEEA-3 glycolipid) and DBA/2 mice. Galactosyltransferase was assayed at 37C in presence of globoside (30μg) and CHAPS (0.5%). The incubation medium contained MnCl2 (5mM), NADH (1mM) UDP-galactose (3.1μM) and MOPS or MES buffer (50mM) in a total volume of 100μl. The lipid soluble radioactivity was measured and enzyme activity expressed as picomoles of galactose transferred/mg/h. In both strains, the specific activity of the enzyme is 2-3 fold greater in the males than in the females. However, kinetic data indicate no significant sex dependent difference in the apparent Km for globoside. Maximal activity of the enzyme in microsomes is obtained at pH 5.6 for female and at pH 5.9 for male kidney. CHAPS and sodium cholate activate but Triton X-100 and Triton CF-54 inhibit the enzyme activity. Pre-incubation of the microsomes at 55C for 1 minute results in 60-70% loss of enzyme activity. However, longer time of pre-incubation results in slightly different rates of inactivation of the enzyme from male and female kidneys. Results of characterization of the reaction product and other kinetic experiments in progress will also be presented.**

**Supported in part by PHS grants NS 15037 and HD 05515**

## 214

OLIGOSACCHARYLTRANSFERASE: N-GLYCOSYLATION OF SYNTHETIC PEPTIDES AND CHARACTERIZATION OF THE GLYCOPEPTIDE PRODUCTS. Richard S. Clark, Shyamal Banerjee, and James K. Coward, Departments of Medicinal Chemistry and Chemistry, The University of Michigan, Ann Arbor, Michigan 48109-1065

The product of the reaction catalyzed by yeast oligosaccharyltransferase was examined in order to determine the nature of the chemical linkage between the sugar and peptide. Biosynthetic donor [$^3$H]lipidoligosaccharide was prepared and used as substrate for yeast oligosaccharyltransferase along with a fully characterized, chemically synthesized, peptide acceptor, N-benzoyl-Asn-Leu-Thr-$NH_2$. The glycosylated peptide product of the *in vitro* reaction was isolated and hydrolyzed with endo-β-N-acetylglucosaminidase-H to remove the large oligosaccharide and leave the glycotripeptide, N-benzoyl-Asn(GlcNAc)-Leu-Thr-$NH_2$. This glycopeptide was purified using gel filtration, affinity binding, and reverse-phase high performance liquid chromatography. The biosynthetic glycopeptide was compared with chemically synthesized glycopeptides in which a 1-amino-GlcNAc moiety was linked to either the α- or β-carbonyl of aspartate. It was determined that the biosynthetic product has the structure in which the carbohydrate is linked to the peptide throught the β-carbonyl of asparagine; i.e., a normal α-peptide. These experiments provide an unambiguous structural proof of the protein-carbohydrate linkage in the glycoprotein product of the oligosaccharyl-transferase-catalyzed reaction.

## 215

SYNTHESIS OF A MODIFIED SUBSTRATE FOR N-ACETYLGLUCOSAMINYLTRANSFERASE-V (GnT V).

Shaheer H. Khan, Saeed A. Abbas*, and Khushi L. Matta.

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The availability of some oligosaccharides that are capable of acting as acceptor-substrates for a single and unique enzyme, even in the presence of other, related enzymes that are invariably found in the same source, is of continuing research interest in our laboratory (K.L. Matta, R. Vig, and S.A. Abbas, Carbohydr. Res., 132 (1984) 119-126; R. Madiyalakan, S. Yazawa, S.A. Abbas, J.J. Barlow, and K.L. Matta, Anal. Biochem., 152 (1986) 22-28; S.A. Abbas, C.F. Piskorz, and K.L. Matta, Carbohydr. Res., 167 (1987) 131-141; R.K. Jain, S.A. Abbas, and K.L. Matta, J. Carbohydr. Chem., 7 (1988) 377-388). In continuance of these studies we have concentrated our efforts on the synthesis of a trisaccharide, 4-nitrophenyl O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→2)-O-(4-O-methyl-α-D-mannopyranosyl)-(1→6)-β-D-glucopyranoside 1, which is intended for use as a specific substrate for the title enzyme. Glycosylation of 4-nitrophenyl 2,3-di-O-acetyl-β-D-glucopyranoside with 2-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-O-acetyl-4-O-methyl-α-D-mannopyranosyl bromide in 1:1 benzene-nitromethane and in the presence of mercuric cyanide afforded a protected trisaccharide that was O-deacetylated to give compound 1.

This investigation was supported by PHS Grant No. CA-35329 awarded by the National Cancer Institute, DHHS.

**216**

AN ELISA ASSAY FOR *N*-ACETYLGLUCOSAMINYLTRANSFERASE-V ACTIVITY Monica M. Palcic[a], Suzanne C. Crawley[a], Gordon Alton[b] and Ole[b] Hindsgaul, Departments of Food Science[a] and Chemistry[b], University of Alberta, Edmonton Canada, T6G 262

*N*-Acetylglucosaminyltransferase-V (GlcNAc-T V) is a key enzyme involved in the branching of Asn-linked oligosaccharides. GlcNAc-T V activity is increased on both polyoma (J.Biol.Chem. (1984) 259:10834) and Rous sarcoma (J.Biol.Chem (1986) 261:10772) transformation.
Expression of GlcNAc-T V activity has also been found to correlate with metastasis (Science (1987) 236:582).

1: βGlcNAc(1->2)αMan(1->6)βMan-O-$(CH_2)_8$COOMe

GlcNAc-T V | UDP-GlcNAc

2: βGlcNAc(1->6) \
αMan(1->6)βMan-O-$(CH_2)_8$COOMe
βGlcNAc(1->2) /

The synthetic trisaccharide **1** has been shown to be a substrate for GlcNAc-T V, producing the tetrasaccharide **2**, thereby providing a convenient radioactive assay for the enzyme (Glycoconj. J. (1988) 5:49). Immunization of a rabbit with the BSA-conjugate of **2** has now produced an antiserum which was passed through an affinity column prepared from **1**. This "refined" antiserum binds to product **2** immobilized on microtiter plates but does not cross-react with acceptor **1** on the same plate. Incubation on microtiter plates coated with the BSA-conjugate of **1** with either human serum or milk resulted in the synthesis of product as detected by this refined antiserum in an ELISA assay. Product formation thus detected was linear with both enzyme concentration and time. Less than a picomole of product per well could be quantitated.

**217**

**PURIFICATION AND PROPERTIES OF UDP-GlcNAc: DOLICHYL-P GlcNAc-1-P TRANSFERASE FROM PIG AORTA.** **K. Ravi and Alan D. Elbein, Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas.**

**The enzyme UDP-GlcNAc:Dolichyl-P GlcNAc-1-P transferase (GlcNAc-1-P transferase) is an important enzyme that initiates dolichol cycle reactions during the biosynthesis of asparagine linked glycoproteins. Although this enzyme has been solubilized and purified from several sources, not much is known about its structure and there is almost no information on whether this enzyme is subject to any type of control. In order to better understand the details of the "Dolichol-Mediated" Pathway, we have purified the GlcNAc-1-P transferase to near homogeneity from pig aorta microsomes using ammonum sulfate fractionation, DEAE-cellulose, Blue Sepharose CL-6B, and hydroxyapatite chromatography. On native gels, the enzyme activity was associated with a high molecular weight band. This high molecular weight band gave rise to three bands with approximate molecular weights of 37 Kd, 52 Kd and 60 KD when run on SPS PAGE under reducing conditions. The purified enzyme was devoid of activity in the absence of phospholipids but activity could be restored by the addition of phosphatidylglycerol and phosphatidylcholine. Although GDP-mannose had no effect on the purified enzyme, dolichyl-P-mannose stimulated the enzyme several-fold when tested immediately after purification of the enzyme. However, this stimulation decreased as the enzyme underwent "aging". The enzyme required divalent cations such as $Mn^{++}$ or $Mg^{++}$ for activity, with optimum concentration of $Mg^{++}$ being about 10mM. The pH optimum for the enzyme was about 7.4 to 7.6. The GlcNAc-1-P transferase, after elution from hydroxyapatite was quite unstable at 4° but fairly stable at -70° for at least several days. This enzyme preparation has been used in a variety of studies and has also been injected into mice to produce monoclonal antibodies. Antiserum from a mouse injected with a partially purified transferase preparation removed the enzyme from solution in the presence of Staphylococcus aureus. We are now screening the hybridomas from this mouse spleen for monoclonal antibodies.** **(Supported by NIH Grants HL 17783 and HL 26890).**

## 218

**PURIFICATION AND PROPERTIES OF DOLICHYL-P-MANNOSE: $Man_5(GlcNAc)_2$-PP-DOLICHOL α-MANNOSYL TRANSFERASE.** **C.B. Sharma, G.P. Kaushal and Alan D. Elbein, Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas.**

**Although the general sequence of reactions of the dolichol pathway for the N-glycosylation of proteins has been delineated, the detailed enzymology and the regulation of this pathway still remain to be determined. Synthesis of the $Man_6(GlcNAc)_2$-PP-dolichol intermediate from Dolichyl-P-mannose (donor substrate) and $Man_5(GlcNAc)_2$-PP-dolichol (acceptor substrate) is thought to be a potential control point in the synthesis of the lipid-linked tetradecaoligosaccharide, $Glc_3Man_9(GlcNAc)_2$-PP-dolichol. The α-mannosythansferase (2.4.1.130) that catalyzes this reaction was solubilized from pig aorta microsomes with 0.25% NP-40, and purified at least 1500-fold using $(NH_4)_2SO_4$ fractionation, DEAE cellulose ion exchange chromatography, Sephacryl S-300 gel filtration chromatography and chromatography on columns of hydroxyapatite. The partially-purified enzyme showed a pH optimum of 6.5 and had a requirement for $Ca^{++}$ for maximum activity. $Mn^{++}$ was only 20% as effective as $Ca^{++}$ and $Mg^{++}$ was inhibitory. The mannosyl transferase activity was also inhibited by the addition of EDTA to the enzyme, but this inhibition was fully reversed by the addition of $Ca^{++}$. The enzyme exhibits a high donor substrate specificity for Dolichyl-P-mannose and was found to be totally inactive with GDP-mannose, Dolichyl-P-glucose, UDP-GlcNAc, GlcNAc-PP-dolichol and $(GlcNAc)_2$-PP-dolichol. Km values for Dolichyl-P-mannose and $Man_5(GlcNAc)_2$-PP-dolichol were 1.8 μM and 1.6 μM respectively, with Vmax values of 0.75 $nM/min^{-1}/mg$ and 29.2 $pM/min^{-1}/mg$. On Biogel P-4 and HPLC columns, the radiolabeled oligosaccharide formed during incubation of Dolichyl-P-[$^{14}C$]-mannose and unlabeled $Man_5(GlcNAc)_2$-PP-dolichol with purified transferase behaved like $Man_6(GlcNAc)_2$. This octasaccharide was susceptible to digestion by endoglucosaminidase H indicating that the newly added mannose was added to the 6-linked mannose in an α1,3 linkage. This linkage was further confirmed by acetolysis of the oligosaccharide product which gave a labeled disaccharide as the major product (>90%). Additional studies on purification and the preparation of antibody against this mannosyl transferase are in progress.** **(Supported by NIH Grant HL-17783 and a grant from the Robert A. Welch Foundation).**

## 219

CHANGES IN PROPERTIES OF MEMBRANE-ASSOCIATED UDP-GlcNAc:DOLICHOL PHOSPHATE GlcNAc 1-PHOSPHATE TRANSFERASE BY NONBILAYER-FORMING PHOSPHOLIPIDS. N.C. Chandra and R. K. Bretthauer. Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556.

The GlcNAc 1-phosphate transferase of the dolichol cycle has been previously reported to be activated by phosphatidylglycerol (PG) when assayed in rat liver microsomes in the presence of a high detergent concentration (0.7% Triton X-100). Activation by PG is not observed when microsomal enzyme activity is measured in the presence of a low detergent concentration (0.005%). Under the latter condition, cardiolipin (CL) and phosphatidylethanolamine (PE), phospholipids known to form nonbilayer, hexagonal phase structures, each have marked stimulatory effects (4-5 fold) on the rate of the enzyme catalyzed reaction. Latency of glucose 6-phosphate phosphatase towards mannose 6-phosphate hydrolysis is not lost under these conditions, indicating that PE and CL are not disrupting the vesicles or inducing membrane leakiness. Kinetic studies with varying CL and dolichol phosphate (dol-P) concentrations show that the apparent Km for dol-P decreases from >30 μg/0.1 ml in the absence of CL to <2 μg/0.1 ml in the presence of saturating CL. Enzyme activity in the presence of CL becomes more sensitive to certain inhibitors as indicated by a 2-3 fold decrease in the concentration of tunicamycin that is required to give 50% inhibition. However, at constant dol-P concentration and in the presence of CL, a 3-4 fold higher concentration of amphomycin is required to give 50% inhibition. Preliminary experiments indicate that enzyme activity also becomes more susceptible to protease inactivation in the presence of CL. These studies suggest that CL and PE are locally disrupting the bilayer structure of the native membrane so as to allow entry of, and exposure of the the enzyme to, the substrate dol-P. The enzyme may thus also become more exposed to the other probes (tunicamycin and protease). Supported by NIH grant HL 33875.

# Biosynthesis of Complex Carbohydrates

## 220

SYNTHESIS OF MUCIN BY RABBIT TRACHEAL CELLS IN CULTURE. S.N. Bhattacharyya, B. Manna and M. Lund. Department of Clinical Investigation, William Beaumont Army Medical Center, El Paso, TX 79920

Rabbit tracheal epithelial cells were isolated by proteinase digestion and grown on collagen-coated dishes in serum-free medium. The cells grown in primary culture incorporated [$^3$H] glucosamine into the secreted material which, when chromatographed on Sepharose 2B column, appeared in the void volume, indicating that this material was represented by high Mr component(s). When the void volume peak was digested with hyaluronidase and rechromatographed on the same column, the peak appeared unchanged in the void volume, suggesting that the peak material did not contain glycosaminoglycans. Alkaline borohydride treatment of the secreted material resulted in loss of sugar components, indicating that carbohydrate residues are linked to the peptide backbone via o-linkage. The overall compositional analysis of this material was similar to those represented by mucin. The presence of collagen on cultured dishes and different growth factors in the culture medium seemed to enhance the production of mucin. Thus, the isolated epithelial-cell culture system can be utilized to answer questions regarding control in production of mucin at the cellular level.

## 221

REGULATION OF TUMOR-ASSOCIATED HYALURONATE: NEW ADVANCES IN THE PURIFICATION OF A HYALURONATE STIMULATORY FACTOR.
Raymond E. Nemec and Warren Knudson, Departments of Biochemistry and Pathology, Rush-Presbyterian St. Luke's Medical Center, Chicago, IL 60612

The glycosaminoglycan hyaluronate is enriched in the extracellular matrix associated with several highly invasive tumors. This increased hyaluronate is often produced by stimulated normal connective tissue cells (e.g., fibroblasts), adjacent to the tumor. We have shown that several human carcinoma cells in culture in fact have the capacity to significantly stimulate hyaluronate synthesis by normal fibroblasts. This stimulation is due to increased levels of fibroblast hyaluronate synthase and reflects an increase in actual mass amount of hyaluronate. The hyaluronate stimulatory activity associated with the human lung carcinoma cell line LX-1 has been most studied. This hyaluronate regulatory factor is not secreted by these cells but is present on the tumor cell surface thus requiring cell-cell contact for stimulation to occur. The stimulatory factor has been solubilized from the membranes in an active form with a variety of detergents and determined to be protease sensitive, heat labile and highly lipophilic. In current studies, in order to effectively purify the hyaluronate stimulatory factor the detergent-solubilized membrane proteins were further dispersed by treatment with 2.5% butanol in phosphate buffered saline containing 1-6 M guanidinium hydrochloride. These conditions effected the dispersion of high molecular weight membrane protein aggregates and allowed true sieving by gel filtration. Sieved proteins were shown to be truely dissaggregated by their rechromatography on gel filtration columns and by SDS polyacrylamide gel electrophoresis. Following removal of the dispersive solvents via dialysis, hyaluronate stimulatory bio-activity was retained and the factor localized to a narrow range of bands between 12,000 and 50,000 daltons.

Supported in part by NIH Grants CA42614 (WK) and NIH SCOR AR39239.

222

MEMBRANE-BOUND UDP-GLUCURONATE CARBOXY-LYASE IN HUMAN PLACENTA.
W. Thomas Forsee, John S. Schutzbach, K. Varughese John, and Helmut Ankel. The Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294, and The Department of Biochemistry, The Medical College of Wisconsin, Milwaukee, Wisconsin 53233.

The biosynthesis of xylose, which occurs through the decarboxylation of UDP-glucuronate, has received scant attention in mammalian tissues. UDP-glucuronate carboxy-lyase activity has been demonstrated in human placenta, which is rich in connective tissue proteoglycans. Ninety percent of the activity can be sedimented by centrifugation, indicating that most of the placenta UDP-glucuronate carboxy-lyase is membrane-bound, in contrast to plant and yeast activities, which are soluble. The membrane-bound enzyme is allosterically activated by UDP-glucuronate. Hill coefficients of 1.7 and 1.9 respectively were observed in the presence or absence of 2 mM NAD. The activity was solubilized in Nonidet P-40 and purified by DEAE-cellulose chromatography, resulting in a 10 fold increase in specific activity. The purified enzyme is active in the absence of exogenous NAD, and is allosterically stimulated by added NAD. In one case the purified enzyme demonstrated hyperbolic kinetics with increasing UDP-glucuronate concentrations, while other preparations were allosterically activated by UDP-glucuronate, both in the presence and absence of NAD. Few investigations have been made of the effect of phospholipids on membrane-bound allosteric enzymes, but the allosteric modulation of 3-hydroxybutyrate dehydrogenase by lecithin (Rudy et al. 1989, Biochemistry 28, 5354), suggests that this is an area of considerable importance. (Supported by NIH Grants CA16777 and GM38643).

223

TIME COURSE OF INCORPORATION OF [$^{14}$C] MEVALONOLACTONE INTO POLYPRENOL AND DOLICHOL IN ISOLATED RYE EMBRYOS.
Robert T. Rymerson and Kenneth K. Carroll, Department of Biochemistry, University of Western Ontario, London, Ontario, CANADA, N6A 5C1.

Secale cereale (rye) is a monocotyledonous plant whose seeds contain both polyprenol ($\alpha$-unsaturated) and dolichol ($\alpha$-saturated). Analysis by HPLC showed that isolated rye embryos contain 198±4 ug/g of these isoprenoids; 67% is polyprenol and the remainder is dolichol. Therefore, these embryos are useful for studying the final steps in the biosynthetic pathway of polyisoprenoids. Studies on germination of rye embryos in $^3H_2O$ showed that, at 20 h, the specific activity of dolichol was 2-fold higher than that of polyprenol. Dolichol and polyprenol may arise from independent biosynthetic pathways, or polyprenol may be converted to dolichol. In an attempt to clarify this, isolated rye embryos were germinated in tap water at 25°C for 2 h and were then incubated with [$^{14}$C] mevalonolactone for 1 h. The embryos were subsequently washed and incubated in 1 mM unlabelled mevalonolactone for various periods of time up to 48 h, saponified and extracted. The incorporation of label into polyprenol was high initially, increased to a maximum value, and then decreased over time. Label was incorporated into dolichol more slowly at first but increased over time to a higher level than that seen in polyprenol. This information suggests a possible precursor-product relationship between polyprenol and dolichol but further studies are needed for confirmation. (Supported by the Natural Sciences and Engineering Research Council of Canada).

Molecular Biology of Glycoconjugates

224

**CHARACTERIZATION OF THE HUMAN GENE THAT ENCODES THE PEPTIDE CORE OF SECRETORY GRANULE PROTEOGLYCANS (PG), AND ANALYSIS OF THE TRANSLATED PRODUCT IN PROMYELOCYTIC LEUKEMIA HL-60 CELLS.** C.F. Nicodemus, S. Avraham, K.F. Austen, and R.L. Stevens. Dept. Med., Harvard Medical School, Boston, MA 02115

A human promyelocytic leukemia HL-60 cell-derived cDNA (cDNA-H4) had been proposed [Stevens *et al.* (1988) *J. Biol. Chem.* 263:7287] to encode the peptide core of a PG that resides in secretory granules of human hematopoietic cells because it encodes a 17.6 kD protein with an unusual 18 amino acid serine-glycine rich glycosaminoglycan region. Based on the deduced amino acid sequence of this cDNA, a 16 amino acid peptide was synthesized and used in the present study to elicit rabbit antibodies that immunoprecipitated an ~20 kD protein from HL-60 cells that had been labeled for 2-10 min with [$^{35}$S]methionine. Because ~150 kD [$^{35}$S]PGs were also immunoprecipitated from HL-60 cells that had been labeled for a longer period of time with either [$^{35}$S]methionine or [$^{35}$S]sulfate, it was concluded that cDNA-H4 does indeed encode a secretory granule PG peptide core. A human genomic library was therefore probed under conditions of high stringency with cDNA-H4 to determine the exon/intron organization of this human PG peptide core gene. The human gene spans ~15 kilobases and consists of 3 exons. The first exon encodes the 5' untranslated sequence of the mRNA transcript, as well as the entire 27 amino acid signal peptide of the translated molecule. The second exon encodes a 49 amino acid region of the peptide core which would be predicted to be the N terminus of the molecule after it's processing in the endoplasmic reticulum. The third exon encodes the remainder of the molecule, including its glycosaminoglycan-attachment, serine-glycine repeat region. As assessed by S1 nuclease mapping analysis, the transcription-initiation site in HL-60 cells for this gene resides 53 bp upstream of the translation-initiation site. When the 500 bp 5' flanking region of this human gene was compared to the corresponding 5' flanking region of the analogous mouse gene, a 119 bp region that immediately precedes the transcription-initiation site was found to be nearly identical. The observation that this nucleotide sequence is more highly conserved than any 119 bp region of the gene that is translated into protein implies that this 5' flanking region contains *cis* regulatory elements that are critical for expression of this gene in hematopoietic cells.

225

ISOLATION AND SEQUENCE ANALYSIS OF A FULL-LENGTH cDNA ENCODING RAT LIVER ALPHA-L-FUCOSIDASE. K.J. Fisher and N.N. Aronson, Jr. Department of Molecular and Cell Biology, PENN STATE UNIVERSITY, University Park, PA 16802.

cDNA clones for alpha-L-fucosidase have been isolated from a rat liver lambda gt11 expression library using both monospecific, polyclonal antibodies against the affinity purified enzyme and biotinylated rat liver fucosidase cDNA sequences as probes. The largest clone contained a 1522-base pair full length cDNA insert (FC9) which encoded the 434 amino acid subunit ($M_r$ = 50,439) for rat liver fucosidase. A putative signal peptide 28 amino acid residues in length preceeded the sequence for the mature protein. In addition, FC9 specified for 11 nucleotides of 5' untranslated sequence, 78 nucleotides of 3' untranslated sequence, and a poly(A) tail. The deduced amino acid sequence from FC9 in conjunction with the experimentally determined amino terminus of the mature enzyme suggested that rat liver fucosidase did not contain a pro-segment. However, there was the possibility of limited amino terminal processing (1-5 amino acids) having occurred after removal of the predicted signal peptide. Amino acid sequences deduced from FC9 were colinear with amino acid sequences measured at the amino terminus of purified fucosidase and on two of its cyanogen bromide peptides. An unusual aspect of rat liver fucosidase protein structure obtained from the FC9 data was its high content of Trp (6%). The coding sequence from FC9 showed 82% homology to that from a previously reported incomplete human fucosidase sequence [O'Brien *et al.* (1987) *Enzyme* **38**, 45-53.]. A cDNA (AllH2) from cAMP stimulated *D. discoideum* was recently found to have sequence homology to human fucosidase [Muller-Taubenberger *et al.* (1989) *FEBS Lett.* **246**, 185-192]. Comparison of rat alpha-L-fucosidase to the protein coded by the *D. discoideum* clone showed 40% of their amino acid sequences to be identical.

## 226

EXPRESSION OF ENZYMATICALLY ACTIVE HUMAN β1,4-GALACTOSYLTRANSFERASE IN *E. COLI*. D. Aoki, M.N. Fukuda and H.E. Appert. La Jolla Cancer Research Foundation, La Jolla, CA 92037 and Department of Surgery, Medical College of Ohio at Toledo, Toledo, OH 43699.

Recently, we have isolated cDNAs for human β1,4-galactosyltransferase, which transfers galactose from UDP-galactose to terminal N-acetylglucosamine in glycoproteins and glycolipids (1). In order to establish unequivocal evidence to show that the cDNA, in fact, expresses a protein having the expected enzymatic activity, we constructed an expression vector pIN-GT (2) for soluble form of the enzyme from the cDNAs and pIN-III-*ompA*2 expression vector (3). Upon induction with isopropyl-β-D-thio-galactoside, fusion protein of *ompA* signal peptide and galactosyltransferase was produced by *E. coli* SB221 cells transformed by pIN-GT plasmid. The enzyme was secreted into bacterial periplasmic space and culture medium. This expression was detected immunochemically by Western blot using anti-galactosyltransferase antibodies as well as by activity assay for galactosyltransferase. The recombinant β1,4-galactosyltransferase was purified to homogeneity by N-acetylglucosamine-Sepharose affinity chromatography. $NH_2$-terminal peptide sequence of purified galactosyltransferase confirmed the cleavage of the fusion protein by bacterial signal peptidase. A HPLC analysis of the oligosaccharide products, which were synthesized by the recombinant β1,4-galactosyltransferase, showed that this enzyme transferred galactose from UDP-galactose to N-acetylglucosamine of acceptor oligosaccharides. The HPLC profile of the oligosaccharide products was indistinguishable to that obtained by human milk β1,4-galactosyltransferase. These results demonstrate that the *E. coli* cells transformed with pIN-GT plasmid produce enzymatically active recombinant human β1,4-galactosyltransferase.

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## 227

CONTROL OF GLYCOPROTEIN SYNTHESIS. ISOLATION OF cDNA CLONES CODING FOR HUMAN UDP-N-ACETYLGLUCOSAMINE:α-3-D-MANNOSIDE β-1,2-N-ACETYLGLUCOSAMINYLTRANSFERASE I

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UDP-GlcNAc:α-3-D-mannosideβ2-GlcNAc-transferase (GnT-I) catalyzes an essential first step in the conversion of high mannose to hybrid and complex N-glycans, i.e., the addition of GlcNAc to (Manα1-6[Manα1-3]Manα1-6)(Manα1-3)Manβ1-4GlcNAc-R to form (Manα1-6[Manα1-3]Manα1-6)(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAc-R. Recently, rabbit liver GnT-I has been purified to homogeneity (Nishikawa et al., *J.Biol.Chem* . **263**, 8270, 1988). Peptide sequence data was used to design oligonucleotide primers which made possible the *in vitro* amplification of cDNAs representing 50% of the coding sequence of GnT-I from rabbit liver mRNA (Sarkar et al., abstract submitted to the Xth International Symposium on Glycoconjugates, Jerusalem, 1989). In this study, a radioactively labelled RNA transcript of one of these cDNAs (0.5kb) was used to isolate a number of human cDNAs from a retinoblastoma library in λgt11. Sequencing of the human cDNAs is presently being carried out and the GnT-I gene is being mapped to the human genome.

(Supported by the MRC of Canada.)

# Glycoconjugates in Development

## 228

**REGULATION OF GLYCOSPHINGOLIPID SYNTHETIC ENZYMES DURING MUSCLE CELL DIFFERENIATION IN VITRO.** K.C. Leskawa and L.D. Cambron, Department of Anatomical Sciences and Neurobiology, School of Medicine, University of Louisville, Louisville, KY 40292.

As single myoblasts contact and fuse to form multinucleated myotubes in vitro there is a transient, increased synthesis of total neutral glycolipids and/or gangliosides during contact and membrane fusion (Leskawa and Hogan, J. Neurochem. 49S:59B, 1987). Using [$^3$H]-Ser as a precursor, clonal murine myoblasts (E63) showed similar increased synthesis during fusion (Cambron et al., Gycocon. J., 5:303, 1988). To better understand regulatory mechanisms involved, glycosyltransferases were assayed during myogenesis of E63 cells, and compared to a fusion-defective varient (fu-1), derived from the same parental cell line (L8). With normal, E63 cells, maximal activation of different glycosyltransferases during differentiation fell into three groups: **(1)** LacCer synthase (GalT-2) and $LcOse_3Cer$ synthase (GlcNAcT-1) activities sharply increased at cell contact and then decreased during membrane fusion; **(2)** during myoblast membrane fusion GlcCer synthase (GlcT) and GM3 synthase (SAT-1) increased and then decreased as myotubes formed; and **(3)** other enzymes gradually increased as myotubes matured, including $GbOse_3Cer$ synthase (GalT-6) and GM1 synthase (GalT-3). The fusion-defective varients, fu-1, showed similar changes with two notable exceptions: the contact-related increase in LacCer synthase was absent and GM3 synthase increased 2 days earlier than that seen with E63 cells (at contact rather than membrane fusion). The results support suggestions that LacCer is involved in cell contact, and further demonstrate that glycolipid synthesis is under complex regulation during differentiation. Supported by NIH grant NS 21057.

## 229

**MEMBRANE DISTRIBUTION OF NEURAL CELL ADHESION MOLECULE DURING NEURITOGENESIS PROMOTED BY GANGLIOSIDE GM1.** H. Safferstein and F.J. Roisen. Dept. of Anatomical Sciences and Neurobiology, University of Louisville, School of Medicine, Louisville, KY 40292.

Previous studies in our, and other, laboratories have shown that gangliosides exert diverse actions on neuronal differentiation. Our localization studies on the ganglioside GM1, following incorporation and neuritogenesis, reveal label along the plasmalemma suggesting the neuronal cell membrane as a focal point for ganglioside effects followed by the activation of second messenger systems. This study addressed how cytoskeletal reorganization, during differentiation caused by GM1 exposure, results in a redistribution of the neuronal cell adhesion molecule (NCAM). Exposure of Neuro-2a neuroblastoma cells to exogenous GM1 causes extensive proliferation of neurites with highly organized cytoskeletal cores of microtubules and microfilaments as revealed by transmission electron microscopy. There was a concomitant redistribution of NCAM under these same neurotrophic conditions, producing a characteristic pattern of linear arrays on the neuronal surface. To probe the cytoskeletal basis of this NCAM redistribution, we utilized various agents. Exposure of Neuro-2a to cytochalasin D (microfilaments), colcemid (microtubules), or taxol (microtubule stabilization and promotion) disrupted the surface topography of NCAM produced solely by GM1. The simultaneous addition of cytochalasin D and taxol augmented the linear distribution of NCAM seen in cultures grown in GM1. This study indicates that microfilaments exert some restraints on microtubule-directed NCAM distribution during ganglioside-mediated neuritogenesis Supported by NIH grant NS24524.

230

ETHANOL DOES NOT INHIBIT NEURITOGENESIS PROMOTED BY EXOGENOUS GANGLIOSIDE GM1, NOR ITS MEMBRANE INCORPORATION, BUT DOES INFLUENCE NEUROBLASTOMA-SUBSTRATUM ATTACHMENT. K.C. Leskawa, G.H. Jackson and K.A. Webster, Department of Anatomical Sciences & Neurobiology, University of Louisville, Louisville, KY 40292.

Others have reported that when ethanol is added to growth media of primary neurons, attachment to substrata is not affected, nor is viability, but the neurons cannot sprout and extend neurites (Dow and Riopelle, Science 228:591, 1985). Since exogenous GM1 is known to promote neuritogenesis, we explored the effect of ethanol upon this neuronal differentiation process. When Neuro-2A cells were cultured in varying concentrations of ethanol for 24 hr, their response (neurite extension) to exogenous GM1 (250 ug/ml) did not vary. Also, ethanol did not inhibit neuritogenic differentiation in response to dibutyryl-cyclic AMP. We have recently reported that the membrane incorporation of exogenous GM1 is mediated by neuroblastoma membrane protein (Leskawa et al., Neurochem. Res., 14:547, 1989). This process, in addition, was not inhibited by ethanol, at concentrations up to 1%.

In contrast, ethanol did influence Neuro-2A cell attachment to a collagen substratum in a dualistic manner. When neuroblastoma cells were seeded onto collagen-coated surfaces in the presence of varying concentrations of ethanol, cell attachment was enhanced (acute exposure). However, when cells were cultured in 0.5% ethanol prior to assay for attachment, a marked inhibition was observed (chronic exposure). These results suggest that ethanol insult during fetal alcohol syndrome may not be due to decreased ability to extend neurites, but may be due to altered interaction with basement membrane or surrounding cytoarchitecture, and that the known membrane fluidizing effects of ethanol may interfere with native membrane ganglioside interactions with receptors of the integrin family (Cheresh, Soc. Complex Carb., 49, 1986). Supported by a grant from the Univ. Louisville Graduate School.

231

EFFECT OF PRENATAL COCAINE EXPOSURE ON NEONATE AND MATERNAL BRAIN GLYCOSPHINGOLIPIDS. K.C. Leskawa[1], G.H. Jackson[1], C.A. Moody[2] and L.P. Spear[2], [1]Dept. Anat. Sci. & Neurobiol., Univ. of Louisville, Louisville, KY 40292, and [2]Dept. Psychol. and Centers for Develop. Psychobiol. & Neurobehav. Sci., SUNY-Binghamton, Binghamton, NY 13901.

Behavioral dysfunctions in offspring exposed in utero to cocaine have been observed, along with alterations in dopamine systems, but few other biochemical studies have been conducted. Given roles in neuronal maturation, glycosphingolipids were analyzed in whole brains of offspring exposed gestationally to cocaine. Pregnant rats were injected with 40 mg/kg/3cc cocaine or saline on gestational days 8-20. Fostered offspring were sacrificed on postnatal days 1 (P1) and 11 (P11), and whole brain glycosphingolipids examined. Cocaine-exposed offspring exhibited markedly elevated levels of both total gangliosides and neutral glycolipids at P1 ($p<0.0001$). Qualitatively, no differences in ganglioside nor neutral glycolipid patterns were observed between cocaine-exposed and normal neonates following separation by 1D and 2D-HPTLC and HPLC. Surprisingly, ganglioside content of whole maternal brains was also elevated ($p<0.0001$), though less than that observed with the neonate brains. When the maternal brain neutral glycolipid fraction was separated by HPTLC, a unique band was observed in all the cocaine-exposed animals (N=9). Since this compound migrated far below the most complex standard avaliable, it likely contains eight or more sugar residues. It does not appear to be fucosylated, since it did not bind agglutinins AAnA, LTA nor UEA-I upon overlay assay. These elevations are in contrast to those reported following fetal alcohol exposure, where decreases in brain gangliosides have been observed. Biochemical consequences of gestational exposure to cocaine may be far-reaching and not restricted merely to the dopamine system. Supported by DA04478 (L.P.S.) and NS21057 (K.C.L).

232

CEREBELLAR SYNAPTIC JUNCTION COMPLEX CARBOHYDRATES IN THE DEVELOPING MOUSE. H. G. Weinstein* and M. Breen; Research-on-Aging Laboratory, VA Medical Center and Department of Pathology, Chicago Medical School, North Chicago, IL 60064

The distribution of synaptic junction glycosylated macromolecules(SJGM) was studied by preparing synaptosomal fractions from the cerebellum of normal mice at 1, 4, 6, 8, 10, 14, 18, 28, and 59 postnatal(PN) days. The SJGM were identified by interacting nitrocellulose blots, obtained after polyacrylamide gel electrophoresis, with Concanavalin A and Wheat Germ Agglutinin lectins. In normal mice at all ages tested the SJGM had a molecular size distribution of 45 to 200 KD, with a major band appearing at 45 KD. However the total amount of SJGM in early PN development increased from 1 to 18 PN days, and then declined thru the 59th day. Our data indicate that glycosylated macromolecules in normal synaptic junctions may have a role in cerebellar synaptogenesis and maturation. (Supported by the Department of Veteran Affairs) *Current Address: Geriatric/Extended Care Service(18), VA Medical Center, North Chicago, IL 60064

233

THE DIFFERENTIAL BIOSYNTHESIS OF LAMININ SUBUNITS IN MYOGENESIS INITIATED WITH 5-AZADEOXYCYTIDINE. Todd G. Kroll and Raymond W. Ruddon, Program in Cellular and Molecular Biology and Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI 48109.

Laminin is an oligomeric glycoprotein that is synthesized and deposited into the extracellular matrix (basal lamina) of epithelial, endothelial, nerve, fat, and muscle cells. We have studied the biosynthesis of laminin in an *in vitro* developmental system (the C3H10T1/2 mouse embryo fibroblast cell line) which recapitulates the committment and differentiation of mesenchyme-like cells into muscle cells after treatment with 5-azadeoxycytidine. Clonal populations of parent fibroblasts, myoblasts, and myotubes, derived from this system were analyzed for laminin biosynthesis using a radio-labeling and immunoprecipitation protocol. The parent fibroblasts biosynthesized predominantly the B1 (205 kd) and B2 (200 kd) subunits of laminin, while the myoblasts and myotubes biosynthesized the B1, B2, and A (400 kd) laminin chains. The level of A chain synthesis in the myoblasts was 7 to 10-fold greater (as a fraction of total protein synthesis) than in the fibroblasts, and this increase could not be accounted for by differential processing or degradation of the A chain in the fibroblast versus the myoblast cultures. The fibroblast cultures lacked the biosynthesis of the A chain and assembled the B1 and B2 chains into a novel laminin tetramer that migrated at about 850 kd on non-reduced SDS-gels. The myoblast cultures biosynthesized the A chain and assembled the A, B1, and B2 subunits into a trimer that migrated at about 950 kd, the size usually attributed to the well-characterized disulfide-linked laminin molecule. These results illustrate that the biosynthesis of laminin subunits and oligomers is developmentally regulated in C3H10T1/2 myogenesis and that the C3H10T1/2 system should be a useful model system to study the regulated expression of laminin and other extracellular matrix glycoproteins.

# Proteoglycans

## 234

RGD-INDEPENDENT MELANOMA CELL ADHESION TO THE CARBOXYL-TERMINAL HEPARIN BINDING FRAGMENT OF FIBRONECTIN (FN) INVOLVES HEPARIN-DEPENDENT AND INDEPENDENT ACTIVITIES. J.B. McCarthy, A.P.N. Skubitz, Q. Zhao, X-y. Yi, D.J. Mickelson, D.J. Klein* and L.T. Furcht. Departments of Laboratory Medicine/Pathology and *Pediatrics. University of Minnesota, Minneapolis, MN 55455.

The adhesion and migration of cells within extracellular matrices is a central consideration in tumor invasion and metastasis. Cell adhesion to extracellular matrix components such as FN has a complex basis, involving multiple determinants on the molecule which react with discrete cell surface macromolecules. Our previous results have demonstrated that normal and transformed cells adhere and spread on a 33 kD heparin binding fragment which originates from the carboxyl-terminal end of particular isoforms (A-chains) of human FN. This fragment promotes melanoma adhesion and spreading in an arginyl-glycyl-aspartyl-serine (RGDS) independent manner, suggesting that cell adhesion to this region of FN is independent of the typical RGD/integrin-mediated binding. Two synthetic peptides from this region of FN were recently identified which bound $^{3}$H-heparin in a solid phase assay and promoted the adhesion and spreading of melanoma cells (McCarthy, et al., 1988, Biochem. 27:1380). The current studies further define the cell adhesion and heparin binding properties of one of these synthetic peptides. This peptide, termed peptide I, has the sequence YEKPGSPPREVVPRPRPGV and represents residues 1906-1924 of human plasma FN. In addition to promoting RGD-independent melanoma adhesion and spreading in a concentration dependent manner, this peptide significantly inhibited cell adhesion to the 33 kD fragment or intact FN. Polyclonal antibodies generated against peptide I also significantly inhibited cell adhesion to the peptide and to the 33 kD fragment but had minimal effect on melanoma adhesion to FN. Anti-peptide I antibodies also partially inhibited $^{3}$H-heparin binding to FN, suggesting that peptide I represents a major heparin binding domain on the intact molecule. The cell adhesion activity of another peptide from the 33 kD fragment, termed CS1 (Humphries, et al., 1987, J. Biol. Chem, 262:6886) was contrasted with peptide I. While both peptides promoted RGD-independent cell adhesion, peptide CS1 failed to bind heparin, and exogenous peptide CS1 failed to inhibit peptide I-mediated cell adhesion. The results demonstrate a role for distinct heparin-dependent and heparin-independent cell adhesion determinants on the 33 kD fragment, neither of which are related to the RGD-dependent integrin interaction with FN.

## 235

LUNG FIBROBLAST HYALURONIDASE. Phyllis M. Sampson, Albert Giandomenico, Bruce Freundlich, Jacquie Little and Jack A. Elias. Department of Medicine, University of Pennsylvania, Philadelphia, PA 19104-4283

Hyaluronidase-like enzymes capable of endoglycosidically degrading hyaluronic acid (HA) have been described in a number of tissues. However, human skin fibroblasts have been reported to lack hyaluronidase. To determine whether human lung fibroblasts contain hyaluronidase, CCL202 cells were grown to confluence and the pattern of elution of radiolabeled HA from Sepharose CL6B columns was compared before and after incubation with cell extracts or medium. HA degrading activity was present in both cells and medium with detergent being required to extract the cellular activity. The enzyme activity from either cells or medium was optimal at pH 3-4 with no activity above pH 5.0. However, the product of digestion appeared to be high molecular weight polysaccharide (>10 K). Studies were then undertaken to determine the mechanism of this degradation. We incubated unlabeled HA with medium and reduced the high molecular weight digestion product with sodium borotritiide. This product, now labeled on the newly generated reducing end only, was further degraded by testicular hyaluronidase or exoglycosidases and then acid hydrolysed. The behavior of the reduced radiolabeled monosaccharide was followed with ion exchange chromatography. The compound generated by testicular hyaluronidase digestion and acid hydrolysis of the original enzymatic digest was not retained on an anion exchanger and had an elution profile similar to glucosaminitol on cation chromatography. The product of the exoglycosidase digestion behaved in the same way after acid hydrolysis but was not retained by either resin prior to acid hydrolysis. Thus the lung fibroblast enzyme cleaved HA to produce N-acetylglucosamine on the reducing end and like other mammalian hyaluronidases, is an endohexosaminidase. Our studies indicate that lung fibroblasts contain a lysosomal type hyaluronidase. These studies also suggest that this hyaluronidase is unusual in that it does not appear to produce tetrasaccharide as its major digestion product. The large size of this digestion product may preclude the activity being detected by colorimetric measurements of hyaluronidase activity. This may explain the apparent absence of activity in human skin fibroblasts. (Supported in part by HL-08805, HL-41216 and HL-36708)

**236**

STIMULATED REPAIR OF ARTICULAR CARTILAGE FOLLOWING MATRIX PROTEOGLYCAN LOSS. J.M. Williams (1-3), M. Moran (4), E.J-M.A. Thonar (2,3), and R.B. Salter (4). Departments of Anatomy (1), Biochemistry (2), and Medicine (3), Rush Medical College, Chicago, IL and Orthopaedics (4), University of Toronto, Hospital for Sick Children, Toronto, Ontario.

Papain has been utilized as a tool in the study of the repair of articular cartilage. Because continuous passive motion (CPM) facilitates repair of articular cartilage defects we performed studies to determine if CPM would affect the repair process following injection of chymopapain into the rabbit knee. Adult male albino rabbits were injected with chymopapain into one knee. Loss of proteoglycans (PG) from the articular cartilage was documented by measuring levels of keratan sulfate (KS) in serum. After the injection, rabbits were subjected to: (i) free cage activity (FCA), (ii) immediate CPM or (iii) FCA for 2 days followed by CPM. Controls included the contralateral noninjected knees and knees from untreated rabbits. At sacrifice, the femoral condyles were processed for histologic analysis. Articular cartilage from all noninjected knees exhibited normal intense Safranin-O staining indicative of an abundance of matrix PGs. After injection of chymopapain, serum KS levels rose sharply and peaked at 24 hours. Injection of 0.2mg or 2.0mg chymopapain into the knee followed by FCA, caused pronounced loss of PGs by day 2, however by day 9 newly synthesized PGs were noted. By day 21, total loss of PGs and surface disruptions were noted in animals which received 2.0mg chymopapain and FCA. However, the articular surface was intact in animals injected with 0.2mg chymopapain;this was accompanied by increased matrix PGs. Animals which received only 0.2mg chymopapain and immediate CPM had a marked loss of PGs, but an intact articular surface on day 2. In animals which received the higher dose of chymopapain the marked loss of PGs on day 2 was also seen but was accompanied by surface disruptions. In all animals which received either chymopapain dose, 2 days of FCA and then CPM, the articular surface was intact by day 9 and partial replenishment of PGs occurred. By day 21, the articular surface was intact and replenishment of PGs continued in animals receiving both doses of chymopapain. The results from this study indicate that CPM of the knee immediately after chymopapain injection may accelerate articular cartilage injury. However, a period of FCA followed by CPM of a chymopapain injected knee appears to accelerate the spontaneous repair by day 21 in animals injected with 0.2mg chymopapain. In animals receiving the higher dose of chymopapain, FCA followed by CPM prevents the damage seen by day 21 and appears to stimulate repair of the articular cartilage matrix. Supported by the Illinois Arthritis Foundation and grants 1-P50-AR39239 and AG-04736 from the NIH.

**237**

IMMUNOCHEMICAL ANALYSIS OF SKELETAL MUSCLE PROTEOGLYCANS. *D.A. Carrino, D.P. Lennon, J.E. Dennis, and A.I. Caplan.* Case Western Reserve University, Cleveland, Ohio.

Both a large chondroitin sulfate proteoglycan (CSPG) and a small dermatan sulfate proteoglycan (DSPG) are produced by skeletal muscle. Several monoclonal antibodies (McAbs) which were raised by others against cartilage CSPG have been used to analyze the core protein of chick muscle CSPG. One McAb (S103L), which reacts with an epitope in the CS-attachment region of cartilage CSPG core protein, reacts weakly with muscle CSPG in a dot blot assay; a McAb (5C4) to the hyaluronic acid-binding region gives strong reactivity with muscle CSPG. Both of these McAbs show strong immunofluorescence in frozen sections of muscle and localize the muscle CSPG to a pericellular region around myotubes. Several core protein McAbs (1C6, 2A5, and 7D1) which require reduction and alkylation of the epitopes either do not cross-react with chick PGs (2A5), react only with cartilage CSPG (1C6), or react with both cartilage and muscle CSPG (7D1). These results indicate that the core proteins of muscle and cartilage CSPGs are different, but share some similarities. Further analysis of the muscle CSPG core protein was performed with McAb MY-174, which recognizes an epitope on the core protein of a CSPG produced by undifferentiated limb mesenchymal cells. This McAb reacts in a dot blot with muscle CSPG core protein, but not with the core protein of cartilage CSPG. In addition, we have generated a McAb to a core protein epitope of skeletal muscle DSPG. This McAb, which does not react with cartilage CSPG, recognizes a band of apparent molecular weight ~100,000 in a Western immunoblot of untreated samples. The band recognized by the McAb is ~43,000 if the sample is first treated with chondroitinase. In immunofluorescence, the McAb stains many different tissues known to contain small DSPGs. Consistent with this, core protein bands of identical molecular weight are recognized on immunoblots of chondroitinase-treated proteoglycans isolated from leg muscle, pectoral muscle, heart, skin, tendon, and bone. In skeletal muscle, the staining is localized not to the muscle cells, but is confined to the fibrous connective tissue septa between the myotubes. The Western immunoblot and tissue distribution suggest that this McAb reacts with a DSPG of the DSPGII class. (Supported by NIH and MDA)

## 238

RAPID, HIGH PRESSURE LIQUID CHROMATOGRAPHY OF PROTEOGLYCANS

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Although high performance liquid chromatography has been used extensively to characterize the glycosaminoglycan chains of proteoglycans, very few researchers have reported the use of this technology for the separation of intact proteoglycan species. The high molarity denaturing buffers required for proteoglycan disaggregation and separation are often not compatible with the low back pressure limitations imposed by many of the HPLC systems designed for the separation of biological macromolecules. In this study, heparan sulfate and dermatan sulfate proteoglycans, obtained by the metabolic labeling of cultured corneal endothelial cells, were rapidly and completely separated in less than an hour in a high pressure liquid chromatography system. The separation, which used a Dionex BioLC system equipped with a Pharmacia Superloop and a ProPac PA1 column, also effected a greater than 10-fold concentration of the proteoglycans during the separation procedure. All buffers were 8 M in urea, and the back pressures generated during the separation were well below the limit of the system. The pooled fractions from the ion exchange column were subsequently analyzed for glycosaminoglycan composition and molecular size. Bovine corneal endothelial cells synthesize both dermatan and heparan sulfate proteoglycans. The heparan sulfate proteoglycan, which is localized in the cell and matrix fraction, is of large molecule size; a much smaller dermatan sulfate proteoglycan is found in the cell, media, and matrix fractions harvested from corneal endothelial cell cell cultures.

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## 239

PROTEOGLYCAN PROFILES GENERATED THROUGH MULTIPLE IMMUNOBLOTTING. Ralph Heimer and Lawrence Molinaro, Jr. Thomas Jefferson University, Philadelphia, PA 19107.

We have proposed a screening method for PG contained in tissue extracts and biologic fluids (Anal. Biochem. 180, 1989) which dispenses with sample purification, biosynthetic labeling and the tedium of analysis of column effluents. Samples, containing at least 10 ng PG, are analyzed by agarose-acrylamide gel electrophoresis, followed by transblotting to nitrocellulose, immunoblotting with monoclonal antibodies (MAB) and $^{125}$I-labeled secondary antibodies, and finally densitometric scanning of autoradiographs. Individual blots may be reprobed after immersion in a chaotropic thiocyanate solution which removes both primary and secondary antibodies but not PG. We now report the inclusion into the profile of epitopes on PG generated through exposure of the blot to chondroitinase ABC. Three types of chondroitin-SO4 (CS) "stubs" attached to core protein, formed by the action of the enzyme, are recognized by MAB. Since these PG remain on the blot after multiple exposure to chaotropic thiocyanate, it is now possible to obtain additional scans, which can discriminate among CS-PG species appearing as closely spaced or even contiguous bands. Purified PG as well as mixtures can be subjected to multiple scanning. For example an A1A1D1D1 adult bovine nasal cartilage PG (ICN Biochemicals) showed two closely spaced bands. The band of higher mobility contained considerably more epitopes reactive with MAB 5-D-4, which interacts with oversulfated keratan sulfate. Yet, 2 bands seen with a similarly prepared calf articular cartilage PG had identical profiles when scanned with MAB for 5 different epitopes including 5-D-4. For this PG a concentration dependent self-association was established, characterized by prevalence of monomer at 4 ng PG/10 µl, equal amounts of monomer and dimer at 64 ng PG/10 µl and the emergence of oligomers beyond 128 ng PG/10 µl. In another application of the method a complex profile of PG was generated from 1 µl samples of synovial fluid from patients with rheumatic diseases. At least 5 distinctly migrating bands were observed, the slowest band probably of highest molecular weight occurring most frequently in rheumatoid arthritis. This band had high reactivity with MAB 2-B-6 suggesting abundance of CS-4 PG. Profiling of PG through multiple immunoblotting deserves the attention of other investigators. (Supported by Eastern PA Chapter Arthritis Foundation).

240

EFFECTS OF MERCURIC SALTS ON CHONDROITINASE ABC- AND AC-TREATED SWARM RAT CHONDROSARCOMA PROTEOGLYCAN. L. Sundaram#, L. Deloria#, T.R. Oegema, Jr.#*, Depts of Orthopaedic Surgery# and Biochemistry*, Univ. of Minnesota, Minneapolis, MN 55455

Ludwigs *et al.* has demonstrated that mercuric salts can remove the unsaturated uronic acid from hyaluronic oligosacchides and suggested that such salts might be effective in removing unsaturated glucuronic acids from chondroitinase-treated proteoglycans (PGs). This would allow rapid preparations of a wide variety of substrates. Proteoglycans prepared from Swarm rat chondrosarcoma proteoglycans by standard extraction and purification methods were digested twice with chondroitinase ABC (PG-ABC) or ACII (PG-AC) in the appropriate buffers. The isolated PGs were treated with various concentrations of mercuric acetate at different pH's. The optimum of 35 mM mercuric acetate at pH 5.0 was very similar for that found for the hyaluronic acid. After treatment with the mercuric salts, the ABCase PG lost reactivity with monoclonal antibodies to the delta-4 and delta-0 unsaturated disaccharides as monitored by electrophoresis in 4% SDS polyacrylamide gel and transblotting to nitrocellulose. There was an altered chemical composition and decrease in size of the linkage region oligosaccharides generated from the chondroitinase ABC- or AC-treated samples when treated with mercuric salt and β-elimination in 0.05 N sodium hydroxide in the presence of 1 M sodium borohydride when the digests were chromatographed on Bio-Gel P4.

RESULTS: Mercury readily removed the unsaturated glucuronic acid as determined by either antibodies or chemical analysis. There was a decrease in mobility on SDS gels of the mercury-treated samples of either PG-ABC or PG-AC. The PG-ABC, as found previously, had 1 repeating disaccharide on the linkage region and reacted with both the delta-4 and delta-0 antibodies and the size, as determined by chromatography on Bio-Gel P4, was consistent with the 1 repeating disaccharide attached to the linkage region. The chondroitinase ACII-treated material in the presence of phosphate buffer yielded profiles similar to the ABC-treated material, but in the absence of phosphate the last 1 repeating disaccharide was removed leaving only the linkage delta-glucuronic acid. Both PG-ABC and PG-AC samples were equally responsive to treatment with mercuric salts and there was a decrease in the size of the linkage region oligosaccharides. It would appear that mercuric salts were an effective and rapid means of removing unsaturated glucuronic acid from both chondroitinase ABC and ACII proteoglycans in good yields.

Reference:
1. Ludwigs, U., Elgavish, A., Esko, J.D., Meezan, E. and Roden, L. (1987) Biochem. J. 245:795-804.
Supported by NIH AR32372

241

PROTEOGLYCANS FROM CORNEAL SCARS: TISSUE DISTRIBUTION
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Previous studies have shown that scar tissue in comparison to normal cornea contains a mixture of smaller and larger proteoglycans (PGs), less keratan sulfate (KSPG) relative to dermatan sulfate (DSPG), KSPG of smaller charge density, and a large DSPG. In this study ultrastructural localization of the PGs in corneal scars were determined by cuprolinic blue dye (CBD) staining. Specific enzymatic digestion of PGs allowed distinction of KSPG- from DSPG-stained structures. The results indicate 1- and 8-week-old corneal scars contain DSPG in the anterior and posterior regions of the tissue. KSPG, however, is located only in the anterior scar. Although no evidence was found for CBD-stained KSPG in the 2-week-old scar, CBD-stained DSPG was abundant. Loss of KSPG in the wound edge early in the healing process, suggest KSPG may diffuse from the normal tissue into the scar. By the second week of healing KSPG synthesized in the scar is low sulfated, constitutes a small proportion of the total PGs in the tissue, and is not stained by CBD. Chemical and immunohistochemical analysis, however, shows that KSPG is indeed present in the tissue and concentrated in the anterior scar. The results of these studies show KSPG is present in the anterior- but not the posterior-scar during healing of full-thickness corneal wounds. This is indicative of the source of cells involved in the healing process.
Supported by PHS grant EY01199.

242

## ISOFORMS OF CORNEAL KERATAN SULFATE PROTEOGLYCAN.

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Corneal keratan sulfate proteoglycan (KSPG) is the major proteoglycan of cornea, and is also present in lesser amounts in aorta, skin, cartilage, and other tissues. KSPG has been characterized in several reports as heterogeneous in size and carbohydrate composition as well as containing multiple proteins. We find that highly purified bovine corneal KSPG contains two KS-linked core proteins. These are 37 kDa and 25 kDa after chemical or enzymatic deglycosylation and are present in a ratio of about 10:1. Proteoglycan enriched for the smaller core protein has a KS/protein ratio about half that of a fraction enriched for the larger core. The two cores have similar amino acid compositions and cross-react antigenically. Cross adsorption of antisera reveals unique antigenic components in each protein. One dimensional peptide mapping after degradation with V8 protease or CNBr shows unique patterns of fragments for the two cores. Two-dimensional tryptic mapping confirms differences in the primary structures of the two proteins but also suggests sequence similarity since 50-60% of the peptides of the two core proteins co-migrate. Both KS-linked proteins can be detected antigenically in several non-corneal tissues. These data suggest the existence of two KSPG isoforms, containing structurally related core proteins and a significant difference in the amount of keratan sulfate attached to each core protein. Supported by NIH Grant EY00952 (to GWC), and American Heart Association Kansas Affiliate Grant in Aid KS-88-G13 (to JLF).

243

## CORNEAL STROMAL PROTEOGLYCAN BIOSYNTHESIS FOLLOWING DIFFERENT CORNEAL STORAGE CONDITIONS AND TRANSPLANTATION.

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The principal secretory activity of corneal stromal fibroblasts (keratocytes) is the renewal of the two predominant corneal stromal proteoglycans, one a dermatan sulfate type (DS-PG) and the other a keratan sulfate type (KS-PG). These PG's are thought to be important in maintaining the order of stromal collagen fibrils and, therefore, the clarity of the cornea. The biosynthesis of these PG's was studied in cat corneas following storage in organ culture at 37° and storage at 4° for 5 days. Controls were corneal buttons removed prior to corneal transplantation. Corneal buttons were labeled with $^{14}C$ leucine and $^{3}H$ glucosamine, the PGs were extracted in guanidine HCl and CHAPS and subsequently isolated by anion exchange chromatography. The relative proportion of each PG was determined by chromatography on Superose 6 before and after digestion with either chondroitinase ABC (DS-PG) or endo-ß-galactosidase (KS-PG). Feline corneas make slightly less KS-PG than DS-PG. Storage at 4° for 5 days resulted in biosynthesis ~75% of control values for both DS-PG and KS-PG. Culture at 37° resulted in a disproportional drop in KS-PG (35% of control) when compared with DS-PG (95% of control). When organ cultured corneas were used for transplants and followed for 22 days post-operatively, DS-PG biosynthesis returned to within normal limits by 7 days while KS-PG remained significantly depressed (< 15%) for the 22 day period. During the immediate post-operative period corneal clarity was not dependent upon a return of KS-PG biosynthesis to normal levels. (This work was supported, in part, by a grant from the Michigan Eye Bank.

244

RELATIONSHIP OF SULFATION TO ONGOING POLYMERIZATION DURING THE CELL-FREE BIOSYNTHESIS OF PROTEOCHONDROITIN 4-SULFATE BY MICROSOMAL PREPARATIONS FROM CULTURED MOUSE MASTOCYTOMA CELLS. G. Sugumaran and J. Silbert. Veterans Administration Outpatient Clinic, and Harvard Medical School, Boston, MA 02108.

It has not been previously established whether sulfation of proteochondroitin sulfate normally occurs during the process of chondroitin polymerization or following complete formation of the glycosaminoglycan chains. In the former case it would be necessary for sulfation to take place at the same membrane location as polymerization. We have now examined the early synthesis of chondroitin 4-sulfate by a microsomal preparation from mastocytoma cells in order to answer this question of sulfation during the process of chain growth. Microsomes from chondroitin 4-sulfate-synthesizing cultured mouse mastocytoma cells were incubated with UDP-[$^{3}$H]GalNAc, UDP-GlcA, and PAPS for 30 sec at 10$^{o}$ and with UDP-[$^{14}$C]GlcA, UDP-GalNAc and PAPS for 4 h at 37$^{o}$ for synthesis of $^{3}$H- and $^{14}$C-labeled chondroitin. The latter incubation provided approximately 100 times as much product as did the short incubation at 10$^{o}$. Upon chromatography of the isolated labeled glycosaminoglycans on a Sepharose CL-6B column, most of the [$^{14}$C]glycosaminoglycan from the 4 h, 37$^{o}$ incubation was excluded from the column, indicating that this nascent glycosaminoglycan had been polymerized fully. In contrast, the [$^{3}$H]glycosaminoglycan from the 30 sec, 10$^{o}$ incubation was mostly retarded upon co-chromatography on this same column, indicating that the nascent glycosaminoglycan was still growing in size. The labeled fractions representing chondroitin/chondroitin sulfate of varying sizes were analyzed for degree of sulfation by degradation with chondroitin ABC lyase followed by paper chromatography and electrophoresis of the products. Results indicated that the [$^{14}$C]glycosaminoglycan formed in the 4 h incubation was 60-70% sulfated. [$^{3}$H]Chondroitin oligosaccharides and small glycosaminoglycan formed in the 30 sec incubation were also sulfated as much as 20-25%. These results indicate that in this microsomal system sulfation takes place while the nascent chondroitin glycosaminoglycan chains are still actively growing in length, although the sulfation lags somewhat behind the polymerization. This not only establishes a common membrane location for both polymerization and sulfation of the chondroitin, but also clearly demonstrates that the sulfation of chondroitin by these mastocytoma cells ordinarily occurs during the process of glycosaminoglycan polymerization rather than subsequent to completion of the glycosaminoglycan chains.

245

EFFECTS OF SULFATE DEPRIVATION ON THE PRODUCTION OF CHONDROITIN/DERMATAN SULFATE BY CULTURES OF NORMAL AND DIABETIC SKIN FIBROBLASTS. C.K. Silbert, M.E. Palmer, D.E. Humphries and J.E. Silbert. Veterans Administration Outpatient Clinic, and Harvard Medical School, Boston, MA 02108.

Human skin fibroblast monolayer cultures from two normal men, three Type I diabetic men, and one Type I diabetic woman were incubated with [$^{3}$H]glucosamine in the presence of diminished concentrations of sulfate. Although total synthesis of [$^{3}$H]chondroitin/dermatan glycosaminoglycans varied somewhat between cell lines, glycosaminoglycan production was not affected within any line when sulfate levels were decreased from 0.3 mM to 0.06 mM to 0.01 mM. Lowering of sulfate concentrations resulted in diminished sulfation of chondroitin/dermatan in a progressive manner, so that overall sulfation dropped to as low as 20% for one of the lines. Amounts of chondroitin 6-sulfate and chondroitin 4-sulfate were determined by degradation of products with chondroitin AC lyase, leaving dermatan sulfate residues undegraded. Chondroitin ABC lyase was then used to degrade all of the chondroitin/dermatan sulfate. Amounts of dermatan sulfate were then determined by subtracting the amount of $\Delta$Di-4S obtained after degradation by chondroitin AC lyase from the amount of $\Delta$Di-4S obtained after the degradation by chondroitin ABC lyase. Sulfation of chondroitin to form chondroitin 4-sulfate and chondroitin 6-sulfate was progressively and equally affected by decreasing the sulfate concentration in the culture medium. However sulfation to form dermatan sulfate was preserved to a greater degree, so that the relative proportions of dermatan sulfate to chondroitin sulfate increased. Essentially all the non-sulfated residues were susceptible to chondroitin AC lyase, indicating that little epimerization of glucuronic acid residues to iduronic acid residues had occurred in the absence of sulfation. These results confirm the previously described dependency of glucuronic/iduronic epimerization on sulfation, and suggest that sulfation of dermatan can take place with sulfate concentrations lower than those needed for 6-sulfation and 4-sulfation of chondroitin. This in turn suggests (as previously noted by Malmstrom) that epimerization occurs simultaneously or in a linked fashion to sulfation in forming dermatan sulfate. There were considerable differences among the six fibroblast lines in susceptibility to low sulfate medium and in the proportions of chondroitin 6-sulfate, chondroitin 4-sulfate, and dermatan sulfate. However there was no pattern of differences between normals and diabetics.

## 246

EARLY CHANGES IN LUNG TISSUE HYALURONAN (HA) CONTENT IN BLEOMYCIN-INDUCED ALVEOLITIS IN HAMSTERS. B.A. Bray*, P.M. Sampson**, M. Osman#, A. Giandomenico** and G.M. Turino*.

Intratracheal instillation of bleomycin in hamsters initiates a series of events that mimic human interstitial pulmonary fibrosis. The temporal sequence of increased biosynthesis of several matrix components has been studied in this model. However, for HA the published data concern only the period 21-30 days post-injury at which time the HA content is 2.8-fold control values (Karlinsky, J.B. Am. Rev. Respir. Dis. 1982; 125: 85-88). To define the early time course of the increase in HA, hamsters were given either 1 unit bleomycin sulfate in 0.2 ml saline or 0.2 ml saline (control) and randomly selected animals from both groups were sacrificed at days 3, 5, 6, 7, 9 and 17. Glycosaminoglycan fractions were prepared from lung tissue of individual animals and were analyzed for HA (Sampson, P.M., Heimer, R. and Fishman, A.P. Anal. Biochem. 1985; 151: 304-308). By day 3 bleomycin-treated lungs contained 273 $\pm$ 37 (SEM) ng HA/mg dry weight, 3.5 times that of saline-treated lungs. The value at day 6 (893 $\pm$ 173) was significantly different from all other groups except the day 5 group (668 $\pm$ 121). Thus, the maximum HA content was reached 5-6 days after injury and at day 6 it was 14.6-fold the normal value. By day 7, however, the HA content had dropped sharply to 459 $\pm$ 115 ng/mg and then declined gradually to 369 $\pm$ 64 at day 9 and 215 $\pm$ 109 ng/mg at day 17. Since the weight of injured lungs was increased to 2.3-fold the control value at day 6, the total HA content of bleomycin-treated lungs was 30-fold that of saline-treated lungs. The maximum increase in HA content occurs prior to the rise in collagen and elastin biosynthesis in this model of pulmonary fibrosis. This observation in addition to the magnitude of the increase and its abrupt decline suggest that HA may be an important initiating factor for subsequent pathological changes in lung matrix components.

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## 247

SYNTHESIS OF SMALL PROTEOGLYCANS BY HUMAN CHONDROCYTES DERIVED FROM OSTEOARTHRITIC FEMORAL HEAD CARTILAGE. C. J. Malemud and R. S. Papay. Department of Medicine, Case Western Reserve University, Cleveland, Ohio 44106

Hyaline cartilage small proteoglycans (smPG) constitute an apparently separate class of glycoconjugates whose functional role is largely unknown. The smPG are primarily non-aggregating and have been reported to have molecular sizes of 120kDa and 76kDa with significantly larger glycosaminoglycan chains (35kDa) than is found in the aggregating proteoglycans. Human chondrocytes were derived from explant outgrowths of osteofemoral cartilage and subpassaged at high initial cell density (2 x $10^5$/ml). The cells were radiolabeled with either $^{35}SO_4$ or $^{35}S$-methionine. The smPG were studied by isolating the most buoyant fraction (A4) from a CsCl density gradient centrifugation under associative conditions (0.5M GuHCl). The cell fraction (CAF) and medium fraction (M) were studied.

The $K_{av}$ of $^{35}SO_4$-labeled A4 from M was 0.58 on Sepharose CL-2B. The glycosaminoglycan chain size was large ($K_{av}$, 0.35 on Sepharose CL-6B). Two $^{35}SO_4$-labeled proteoglycan subpopulations with $M_r$'s greater than 200 kDa (smPG-I) and approximately 180kDa (smPG-II) were seen on 3-16% gradient slab gels containing SDS. The smPG-II was found primarily in the CAF, whereas the M compartment contained both smPG-I and smPG-II. Chromatography of A4 on DEAE-Sephacel eluted with a NaCl gradient (0.2M-1.4M) showed a single symmetrical peak (0.55M NaCl) consistent with a proteoglycan species enriched in a mixture of chondroitin sulfate/dermatan sulfate. Dot-blot analysis with monoclonal antibody 5D4 confirmed the presence of keratan sulfate-containing proteoglycan in the A4 fraction as well. Osteofemoral cartilage cultured as short-term explant cultures also synthesized smPG-I and smPG-II, indicating that the cultured human chondrocytes were able to recapitulate the smPG repertoire of the tissue.

$^{35}S$-methionine-labeled A4 from the CAF was immunoprecipitated by monoclonal antibody S103L, whereas A4 from M was not. The $M_r$ of the $^{35}S$-methionine-labeled immunoprecipitate on 3-16% gradient slab gel was identical to that of smPG-II. Recently, Kearns et al. (J. Cell Biol. 107:158a, 1988) determined that the S103L epitope for chick proteoglycan monomer resides N-terminal to the C-terminal G3 domain. These data suggest that the smPG of human chondrocytes may be truncated forms of the large proteoglycans also synthesized by these cells. Supported by NIH grant AG-02205.

**248**

CHONDROITIN SULFATE SYNTHESIS PRIMED BY LIPID-LINKED β-D-XYLOSIDES. Fulgentius N. Lugemwa and Jeffrey D. Esko. Department of Biochemistry, Schools of Medicine and Dentistry, University of Alabama at Birmingham, Birmingham, AL 35294.

β-D-xylosides containing small hydrophobic aglycone moieties act as primers for chondroitin sulfate synthesis in many cell types. To examine if β-D-xylosides of naturally occurring lipids would also act as primers, xylosides of 1-octanol, 1-hexadecanol, cholesterol, farnesol, and 2-monooleyl glyceryl ether were synthesized. The priming activity of the xylosides were tested by supplementation of pgsA-745 cells, a mutant Chinese hamster ovary cell line defective in xylosyltransferase. These cells do not synthesize any glycosaminoglycan under normal conditions but make chondroitin sulfate when supplemented with p-nitrophenyl-β-D-xyloside (Esko et al., (1988) J. Biol. Chem. 262, 12189). With the exception of 2-monooleyl glyceryl ether xyloside, all of the lipid-linked xylosides efficiently primed chondroitin sulfate synthesis. When cultured in the presence of fetal bovine serum, the cells secreted the majority of chondroitin sulfate into the growth medium. However, treatment of cells with chondroitinase ABC at 4°C showed that some chains were also associated with the cell surface. Studies are underway to establish whether the lipid moiety remains intact and whether the appearance of the glycosaminoglycan in the medium is due to partitioning between cell membranes, serum lipoproteins, and growth medium. The failure of the 2-monooleyl glyceryl ether xyloside to prime glycosaminoglycan synthesis is interesting since uptake, sorting through intracellular membranes, and metabolism of the xyloside may depend on the nature of the lipid moiety. That lipid-linked xylosides do not prime heparan sulfate chains suggests that the branchpoint enzyme that adds the first N-acetylglucosamine residue during heparan sulfate synthesis requires structural information not present in either soluble or membrane-associated primers.

**249**

THE LEVEL OF HEPARAN SULFATE CHAIN DEACETYLATION IS DEPENDENT UPON THE ACTIVITY OF N-SULFOTRANSFERASE. Karen J. Bame and Jeffrey D. Esko, Department of Biochemistry, University of Alabama at Birmingham, Birmingham AL, 35209.

We have previously characterized a Chinese hamster ovary cell mutant, pgsE-606, defective in the heparan sulfate modification enzyme N-sulfotransferase. PgsE-606 cells only contain 25% of wild-type N-sulfotransferase activity, and the heparan sulfate made by the mutant is undersulfated to the same extent as the enzyme deficiency. These results suggest that the level of N-sulfotransferase activity in the cell determines the extent of heparan sulfate chain modification (Bame and Esko, (1989) J. Biol. Chem. 264, 8059). Since deacetylation is a prerequisite for N-sulfation, wild-type and pgsE-606 cells were analyzed for N-deacetylase activity, and for the level of free amino groups on purified heparan sulfate chains. Both cell types contain comparable N-deacetylase activities, however, when the purified heparan sulfate from each cell line was treated with high pH nitrous acid, wild-type heparan sulfate appeared to have more free amino groups on the chain than pgsE-606 heparan sulfate. An opposite effect is observed when wild-type cells are incubated in the presence of chlorate, a competitive inhibitor of PAPS formation. Wild-type heparan sulfate, purified from chlorate treated cultures is extensively undersulfated (300 cpm/μg versus 2600 cpm/μg). However, unlike pgsE-606 heparan sulfate, the undersulfated chains contain measurable amounts of free amino groups (27% unsubstituted glucosamine). Preliminary experiments with chlorate treated pgsE-606 heparan sulfate also indicate the presence of free amino groups, but not to the same extent as chlorate treated wild-type. Taken together, these results suggest that the N-deacetylase and N-sulfotransferase reactions are coupled *in vivo*, and the extent of deacetylation is dependent upon the activity of the N-sulfotransferase.

## 250

A STRUCTURAL VARIANT OF THE ANTITHROMBIN III BINDING SITE AND ITS DISTRIBUTION IN HEPARIN FROM DIFFERENT SOURCES. Robert J. Linhardt and Duraikkannu Loganathan. Division of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa City, IA 52242 USA

Heparin is a polydisperse copolymer of 1→4-linked glucosamine and uronic acid residues and has been in clinical use for over fifty years as an anticoagulant. Earlier studies have shown that a specific pentasaccharide sequence in heparin representing the minimal binding site for antithrombin III (ATIII) is largely responsible for heparin's anticoagulant activity. Recently, analysis of porcine, bovine and clam heparins using $HNO_2$ depolymerization led to the proposal of structural variability of ATIII binding site. This variability includes the replacement of 2-acetamido-2-deoxy-α-D-glucopyranose 6-O-sulfate by 2-sulfamido-2-deoxy-α-D-glucopyranose 6-O-sulfate. However, it is unclear from these studies whether L-iduronic acid that precedes 2-sulfamido-2-deoxy-α-D-glucopyranose 6-O-sulfate is 2-O-sulfated. Such 2-O-sulfation is biosynthetically permissible since the adjacent glucosamine is N-sulfated.

As part of our continuing structural studies on heparin, we reported earlier the structure elucidation of a hexasaccharide containing a portion of ATIII binding site which was purified from depolymerized porcine mucosal heparin using flavobacterial heparinase. The isolation and characterization of a tetrasaccharide possessing a hitherto unreported, biosynthetically permissible structural sequence in the ATIII binding site will be presented. Structure elucidation of this tetrasaccharide was mainly based on two-dimensional $^1H$ NMR (COSY and ROESY) and Fast Atom Bombardment Mass Spectrometry (FAB-MS). The potential value of FAB-MS and ROESY in offering sequence information on such oligosaccharides will be discussed. Heparins from a variety of species and tissue sources were examined using oligosaccharide mapping by strong anion exchange HPLC and gradient polyacrylamide gel electrophoresis. This study shows the widespread distribution of the new ATIII binding site structural variant (bovine heparin containing maximum amount) along with the previously reported ATIII binding site in different heparins.

## 251

CHARACTERIZATION OF PROTEINS SUBSTITUTED WITH KERATAN SULFATE FROM BOVINE ARTICULAR CHONDROCYTES

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Proteins substituted with keratan sulfate (KS) have been described in a variety of connective tissues, including cartilage, disc, bone, tendon and cornea. In cartilage, keratan sulfate is known to be present on the large aggregating chondroitin sulfate rich proteoglycan. Recently, it was reported that cartilage also contains lower molecular weight KS rich species (1), which we have shown to be a biosynthetic product of articular chondrocytes in culture (2). We here report on the structural characteristics of these molecules and describe some new aspects of their catabolism by cultured chondrocytes.

Bovine articular chondrocytes at day 2 of culture incorporate between 10 and 15% of $^{35}S$ $Na_2SO_4$ into molecules that can be recovered from CsCl gradient fractions with densities <1.50g/ml. These low buoyant density sulfated molecules can be seperated by salt gradients on MonoQ columns into two major groups, namely KS substituted proteins (0.1M - 0.6M NaCl) and dermatan sulfate proteoglycans (0.6M - 1.0M). Pretreatment of chondrocyte cultures with tunicamycin showed that the synthesis of the small KS substituted proteins was insensitive relative to $^3H$ mannose incorporation, suggesting that the KS on these proteins is largely, if not entirely bound via O-glycosidic bonds. When the KS containing molecules from MonoQ were applied to Superose12 columns or SDS PAGE, evidence was obtained for 3 sizes of molecules with apparent molecular weights > 70K, 25K and 10K. Interestingly, the medium compartment of cultures were enriched in the high molecular weight species, whereas the cell layers contained a higher percentage of the smallest species. $NaBH_4$ treatment of individual species, followed by rechromatography on Superose 12, indicated that the larger species were substituted with KS chains of very similar size (approx 10K). The lowest molecular weight species did however not show any change in elution position after the $NaBH_4$ treatment, suggesting the presence of 'free' KS chains in these preparations. Pulse chase experiments have indicated that the low buoyant density KS substituted molecules are indeed catabolised in these cultures with a $t_{1/2}$ of less than 4 hours. Thus, total macromolecular $^{35}S$ in the low buoyant density KS fractions decreased by 55% in a 4 hour chase, follwed by a slower decrease to 35% of the initial macromolecular label in an additional 20 hour chase. Current experiments are aimed at investigating the possiblity that the heterogeneity in size of the KS substituted proteins might be due to catabolic processing by chondrocytes of a single precursor proteoglycan of about 70K.

1. Funderburgh et al. (1987) J. Biol. Chem. 262, 11634-11640
2. Plaas et al. (1989) J. Biol. Chem. In Press

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252

ANALYSIS OF TRYPTIC FRAGMENTS FROM NEWLY SYNTHESIZED RAT CHONDROSARCOMA PROTEOGLYCAN: Y. Cheng, M.J. Hejna, R.L. Croxen, K. E. Kuettner, and J.H. Kimura. Department of Biochemistry, Rush-Presbyterian-St.Luke's Medical Center, Chicago, IL 60612

Cartilage proteoglycan (PG) aggregates consist of multiple PG molecules bound to individual strands of hyaluronate (HA) in ternary complex with link protein. The aggregation of newly synthesized PG requires an appropriate conformation of the N-terminal HA-binding region (G1), or the most N-terminal of the three globular domains of the core protein. The presence of link protein protects the globular G1 from cleavage by protease. It has been reported that newly synthesized PG binds with lower affinity to HA than material from cartilage tissue. To determine if this lower affinity might preclude the isolation of G1 from newly synthesized PG in link-stabilized aggregates, we have been examining the characteristics of proteolytic fragments produced by trypsin treatment of metabolically labeled PG synthesized by cultured Swarm rat chondrosarcoma cells. PG from cultures labeled for 4 or 8 h with [$^3$H]leu or [$^3$H]ser were purified by dissociative CsCl gradients and reaggregated with carrier aggregate. Trypsin treatment (1/100, w/w) of the aggregate for 6 h followed chondroitinase ABC (ChABC) digestion and SDS-PAGE analysis indicated that about 19% of the leu and 8% of the ser radioactivity in the PG was retained by G1, a value in close agreement with the percentage predicted by the cDNA sequence. The tryptic digest was also subjected to an associative CsCl gradient fractionation. G1 containing material was found largely in the top, A4, fraction, while evidence with a monoclonal antibody (1-C-6) and peptide analysis on reverse phase HPLC indicate that fragments from the G2 domain can be recovered from the A2. Interestingly, a peptide was found in the A1 fraction which could be labeled with [$^3$H]cys and required ChABC treatment to elute as a single peak on HPLC, a result consistent with a predicted tryptic peptide at the juncture of the G3, or C-terminal globular region, and chondroitin sulfate attachment domains of core protein which contains both a cys and a ser-gly. The results indicate that newly synthesized PG from the rat chondrosarcoma is amenable to tryptic analysis and that fragments from the G1, G2, & G3 domains can be separated by CsCl gradients.

253

**ISOLATION AND CHARACTERIZATION OF HYALURONIC ACID (HYALURONAN) FROM HUMAN PULMONARY FIBROSIS.**

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High content of sulfated glycosaminoglycans has been observed in human pulmonary fibrosis. More recently, hyaluronate (hyaluronan) has been shown to increase in several lung disorders, including experimental pulmonary fibrosis induced in hamsters by the use of bleomycin. The present study deals with the characterization of the non-sulfated glycosaminoglycan fraction of human fibrotic lungs which represents more than 50% of the total GAG of that tissue. GAGs were isolated from normal and fibrotic human lungs by digestion with proteolytic enzymes, chloroform-amyl alcohol treatment and subsequent fractionation with graded concentrations of ethanol (28%, 36% and 50% ethanol). Subsequently, the major GAG fractions were chromatographed on ion-exchanger (AG 1x2, Cl form), using increasing concentrations of salt (0.5 to 2.0M NaCl). The results revealed a three-fold increase in a non-sulfated GAG eluted from the column at 0.5M NaCl for material derived from the fibrotic lungs compared to that of normal lungs used in control experiments. Cellulose acetate electrophoresis carried out in pyridine-acetate buffer pH3.1 and HPLC data showed this fraction to be homogeneous. This material was characterized as hyaluronic acid (hyaluronan) on the basis of chemical analyses and thin layer chromotography of its monosaccharide components following acid hydrolysis. In addition, this GAG was found to be completely digestible by Testicular and streptomyces hyaluronidases. Preliminary circular dichroism studies also revealed the identity of this molecule with hyaluronate from rooster comb. The molecular weight of this HA fraction estimated by viscosity was found to be between 0.9 to 1.1 x $10^5$ Based on these findings, its is clear that hyaluronic acid (hyaluronan) plays a significant role in human pulmonary fibrosis as its concentration increases more than three-fold over that of the normal. Such increase in hyaluronan also appears to be evident in GAGs obtained from fibrotic lungs induced by bleomycin.

## 254

**Characterization of Cartilage Chondroitin Sulfate Proteoglycan (CSPG) Core Protein: an Immunochemical and Ultrastructural study.** James E. Dennis, David A. Carrino, and Arnold I. Caplan. Department of Biology, Case Western Reserve University, Cleveland, OH 44106

Core protein-specific monoclonal antibodies were used to probe the protein sub-structure of cartilage CSPG by both immunoblot analysis, and ultrastructural mapping of the monoclonal antibody epitope. For immunoblotting, CSPGs were cleaved with cyanogen bromide (CNBr) for increasing lengths of time, digested with chondroitinase (CSase) and keratanase (KSase) to remove glycosaminoglycans, and the resulting denuded peptides were electrophoresed on 5-17.5% linear gradient gels. These displayed peptides were transferred and probed with monoclonal antibodies specific for the hyaluronic acid binding region (antibody 5C4) and for a region containing chondroitin sulfate (CS) linkages (antibody S103L). 5C4 localized a series of low molecular weight peptide fragments at 34, 29, and 13 KD, while S103L recognized a high molecular weight band at approximately 200 KD. Neither antibody had cross-reactivity to peptide fragments recognized by the other antibody. These results were compared with a previous investigation of the CNBr cleavage peptides of cartilage CSPG core protein which revealed a series of 17 peptide fragments (Haynesworth et al., *J. Biol. Chem.*, 1987, 262:10574-10581). The 200 Kd peptide recognized by S103L corresponds to the largest band which was shown to contain CS-linkages. The peptides recognized by 5C4 may correspond to two non CS-linked peptides of 27 and 15 KD apparent molecular weight, both of which contain disulfide crosslinks. The 34 KD fragment may correspond to a peptide fragment identified as having neither KS- or CS-linkages. These results are consistent with the data which show that the HA binding region is located at the N-terminal globular region which contains disulfide bonds, but has no CS linkages.

In order to precisely define the epitope for S103L, intact CSPG core protein was incubated with affinity-purified antibody, and rotary shadowed for electron microscopy. Images of core protein without antibody revealed a structure containing 3 globular regions (G1, G2, and G3) and two elongated regions (E1 and E2). The N-terminal globular region (G1) is separated from G2 by a short elongated region 21 nm in length (E1). The G3 region is located at the C-terminus, and is separated from G2 by a 105 nm elongated region (E2). Samples incubated with S103L bound antibody on the E2 region approximately 34 nm from the C-terminal globular region (G3). These results have been confirmed, independently, by sequence analysis of cDNA's selected with S103L from a gt11 expression library (Mensch et al., *J. Biol. Chem.*, submitted).

These methodologies will be appplied to the characterization of a muscle CSPG which has been shown to be transiently expressed during embryonic development. Interestingly, this muscle CSPG is reactive with the cartilage core protein antibody, S103L.

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## 255

ABSENCE OF NORMALLY SULFATED KERATAN SULFATE IN CARTILAGE IN TYPE 1 MACULAR CORNEAL DYSTROPHY. E.J-M.A. Thonar, D.P. Edward, M. Srinivasan, M.E. Lenz and O.M. Tso. Rush-Presbyterian-St. Luke's and Univ. Illinois, Chicago, IL.

Macular corneal dystrophy (MCD) is a blinding inherited disease of heterogeneous etiology that is characterized by abnormal deposits within the corneal stroma, keratocytes and the endothelium. In type 1 MCD, the most prevalent form of the disease, the deposits contain a proteoglycan (PG) with unsulfated keratan sulfate (KS) chains. We present direct evidence that normally sulfated KS is not present in the cartilage PGs of a patient with this disease of the cornea. The patient was a 70 year old Asian Indian male with clinical signs of MCD. Normal, sulfated, KS was not found in his serum (less than 2 ng/ml) indicating this individual had type 1 MCD. None of four anti-KS antibodies tested gave positive immunostaining of formalin-fixed sections of cornea and nasal cartilage from this patient. In contrast, all antibodies stained the stroma and keratocytes of normal human cartilage and the extracellular matrix of normal human cartilage. Further, an ELISA-inhibition assay, using the well characterized 1/20/5-D-4 anti-KS antibody, was used to show that papain digests of corneal and cartilage tissues from the MCD patient contained at least 900 times less keratan sulfate epitope than did papain digests of corresponding normal human tissues. The absence of sulfated KS in the cartilage matrix in type 1 MCD suggests the deficiency in sulfotransferase is not restricted to the cornea as previously thought. It also implies a common sulfotransferase is probably involved in the sulfation of KS chains on cartilage and corneal PGs. Because type 1 MCD is not associated with an increased incidence of cartilage disease, our findings also suggest the presence of sulfated KS chains does not appear to be essential for the functional properties of cartilage PGs.

BASE - CATALYSED MODIFICATIONS OF HEPARIN

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A selective intramolecular displacement of the sulfate group of residues of α-L-iduronic acid 2-sulfate (1) in heparin may be induced in alkaline solution. Depending on the reaction conditions used, three different modified forms of heparin are obtained, in which residue 1 is substituted by either 2,3-anhydro-α-L-guluronic acid (2), α-L-galacturonic acid (3), or α-L-iduronic acid (4). Regio- and stereoselective aspects of the ring-opening reactions of 2 to give 3 or 4, as well as analogs of them, are described. Although the formation of these novel glycosaminoglycans is accompanied by only moderate depolymerization, there is a marked decrease in anticoagulant and other biological activities. Cation - binding characteristics of these modified polymers are described in relation to those of heparin.

# Collagens

257

TYPE VI COLLAGEN IN CARTILAGE: OPTIMUM CONDITIONS FOR ITS IMMUNOLOCALIZATION. C.A. McDevitt, J. Kollar, N. Tsumura, J.T. Andrish and R.R. Miller. Department of Musculoskeletal Research, Cleveland Clinic Foundation Research Institute, Cleveland, Ohio 44195

Type VI collagen is a connective tissue matrix glycoprotein comprised of two large globular proteins connected by a relatively short triple helical collagenous domain. We have previously reported that experimental osteoarthritic (OA) cartilage is enriched in this protein. In this study, we systematically examined the conditions for optimum immunolocalization of type VI collagen in cartilage. The effect of formalin fixation, chondroitinase ABC digestion (0.0050/50 $\mu$l for 30 or 120 mins) with and without prior fixation, and streptomyces hyaluronidase digestion (0.10/50 $\mu$l for 120 mins) prior to reaction of the tissues with primary antibody was investigated. Immunolocalization was performed with a monoclonal antibody that recognized the large $\alpha$3 chains of type VI collagen in western blotting and FITC labelled goat anti-mouse IgG (whole molecule). The type VI collagen was predominantly localized around the cells as a discrete ring in normal cartilage, with a diffuse low density fluorescence in the interterritorial matrix. The intensity of fluorescence in the interterritorial matrix was significantly increased when the fixation step was omitted and enzymatic digestion was prolonged for 120 mins. In contrast to the normal cartilage, the type VI collagen surrounded the chondrocytes in the experimental osteoarthritic cartilage as a broad diffuse ring. These studies and the reported presence of an array of RGD peptides in type VI collagen support the hypothesis that this protein is an important constituent of the chondron surrounding chondrocytes and that its distribution is changed in OA. (Supported by NIH grant AR 39569)

258

ISOLATION AND PURIFICATION OF CHICK EMBRYO OSTEOBLASTS BY ALGINATE BEADS.
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Chick embryo osteoblasts elaborate and mineralize the extracellular matrix they produce in vitro. These cells therefore can be used to study the relationship between collagen structure and the biomineralization process. A novel method has been developed to isolate, purify, and propagate primary chick embryo osteoblasts (CEOB). Calvariae of 16 day embryos were dissected and minced, and cell explants consisting of osteoblasts and fibroblasts were grown on plastic surfaces. The cell mixtures were then transferred to alginate polymer beads. Purification of CEOB cultures was achieved because of the growth advantage CEOB cells have over contaminating fibroblasts in the alginate bead environment. The osteoblast phenotype of CEOB cells was determined by biosynthetic, histochemical, and morphological criteria. The phenotype was maintained for > 8 months, which allowed an initial characterization of the collagens and proteoglycans synthesized by CEOB cells. Conditions also have been established to transfect the CEOB cells at a level of efficiency comparable to transfection of chick embryo fibroblasts. It is possible, therefore, to study collagen structure-biomineralization relationships by site-directed mutagenesis of collagen cDNAs and transfection of CEOB cultures.

259

A TRANSGENIC MOUSE MODEL OF THE MILD DOMINANT FORM OF OSTEOGENESIS IMPERFECTA.

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Osteogenesis imperfecta type I (OI I) is characterized by bone fracture without deformity, blue sclerae, normal stature, presenile hearing loss, and autosomal dominant inheritance. Dentinogenesis imperfecta occurs in a small subset of OI I families. Mutations in all cases to date have occurred in the genes which encode type I collagen. Although the molecular basis of this disease is heterogenous, most OI I mutations are thought to be regulatory in nature (i.e. result in a nonfunctional allele). Heterozygous Mov-13 mice (referred to here simply as MOV-13 mice) represent a potential model of OI I in that integration of a murine retrovirus within the first intron of the α1(I) collagen gene effectively blocks transcription and thereby results in a null allele. This study demonstrates that MOV-13 mice are indeed a faithful phenocopy of OI I, having 1). a non-lethal, dominant disorder, 2). a reduced amount of type I collagen in skin, 3). a mixed neurosensory and conductive hearing deficit that is progressive in nature, and 4). bone fragility. The MOV-13 model provides an opportunity to study the effects of the null allele on the assembly, structural integrity, and wound healing properties of mouse connective tissue. The mice also provide a model for therapy designed to strengthen mineralized as well as non-mineralized connective tissues and thereby ameliorate the mutant phenotype.