Glycosylation & Disease

A Fast Publication Journal on Glycosylation

3rd Jenner International Immunoglycobiology Meeting: Abstracts

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SESSION 1: Glycoprotein Analysis

Computer graphics molecular modelling in glycoimmunology: fact or fantasy?

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There are many instances where it is important to have a visual image of a molecule to understand its interactions. X-ray crystallography is a powerful tool for this, but complementary information at the sub-molecular level can be obtained by solution physicochemical methods (NMR, CD, ROA etc) and often only these dynamic methods are possible or desirable. In order to visualise information from the latter we need to input the data into molecular modelling simulations. In the absence of X-ray studies of a particular (glyco)protein (due to inadequate amounts, inability to crystallise or lack of instrument time), computer graphics programs can be used to carry out homology searching and mutation. If no homologous sequences can be found in the database of already crystallised molecules, *de novo* approached can be attempted (particularly incorporating NMR data in an iterative process), but these will be by definition only approximate. However, if they predict experimentally provable hypotheses, their value will have been assured. In the field of glycoimmunology, there are several areas where molecular models have helped with structure/function interpretations. In particular in visualisation of protein glycosylation where this was not detected by electron density or prevented crystallisation and also to study oligosaccharide solution dynamics. Thus molecular mechanics simulations including NOE data from NMR have been used to map the conformations of oligosaccharide tumour and differentiation antigens and ligands for selectins; design inhibitors of oligosaccharide-to-protein interactions; provide support for the possible role of underglycosylated immunoglobulins in pathology; study the effects of oligosaccharide chains and glycan anchors on protein conformation, trafficking and function; and, identify protein motifs exposed away from covalently attached oligosacharide chains which are hence accessible for protein-to-protein or protein-to-carbohydrate interactions.

Chemometrics of oligosaccharide analysis: parameterization of HPLC of PA-oligosaccharides

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For structural identification of minute quantities of oligosaccharides derived from glycoconjugates, chromatography or electrophoresis may be the only methods available. In such methods, oligosaccharides are compared with the known standards under identical conditions. When the scope of analysis is limited to a single glycoprotein such as human immunoglobulin, it may be possible to obtain a complete library of the known oligosaccharides. However, it is impossible to obtain a complete set of oligosaccharides hitherto reported, and often structural assignment must be made without any standards. Attempts have been made to correlate the mobility of the oligosaccharides during chromatography or electrophoresis to their structural features. For pyrydylamine-modified oligosaccharides of Asn-linked type, there are abundance of chromatographic data with which contribution of each sugar to the elution volume can be parameterized. The assumption used is that contribution by each monosaccharide at each position is independent to others. This assumption seems to be held quite well in our analysis. Use of the parameterized values for determination of unknown structures have been successful in a number of cases tested, and it also led to discoveries of new structures.

The different FACEs of disease

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Carbohydrates are involved in a number of pathologic processes and they can be useful predictors of disease. Carbohydrate-associated diseases include metabolic, infectious, autoimmune, and neoplastic diseases. Over the past 20 years, the ability to accurately measure protein analytes in patients has greatly benefitted medical practice. The measurement of carbohydrate analytes in patients should result in better ways to predict disease and manage patients. Since most clinical analytes are present in levels at or below micrograms/ml, conventional isolation and analysis methods cannot be used to measure carbohydrate analytes in human body fluids. We have tested whether a new carbohydrate analytic method called fluorophore-assisted carbohydrate electrophoresis (FACE) might be a useful tool for measuring carbohydrates in human body fluids. The method is sensitive at levels in the picomole range (nanograms/ml) and is rapid and economical. We will present clinical data in several diseases: measurement of urinary oligosaccharides in carbohydrate metabolic diseases, direct measurement of plasma heparin levels for therapeutic drug monitoring, measurement of urinary chondroitin sulfate and glycosyl-lysine in osteoporosis, and the measurement of abnormally glycosylated plasma proteins in patients with alcoholism. We have concluded, based on pilot clinical studies, that FACE warrants further investigation as a clinical tool for the diagnosis of carbohydrate-related diseases and for monitoring therapeutic carbohydrate pharmaceuticals.

New advances in the analysis of carbohydrates on glycoproteins

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We will present the methods used to release glycans from glycoproteins, via the automated hydrazinolysis procedure, and the newly launched fluorescent labelling system used for their detection. Additional data will be provided in the use of these fluorescently labelled glycans in both the size based RAAM™ 2000 GlycoSequencer and a general 2-dimensional hplc separation strategy exploiting the glycans charge and hydrophobicity characteristics. Example chromatograms will be presented to illustrate all three separation methods.

1.1

Study of the O-linked carbohydrate structure of IgAl and secretory IgAl in serum, synovial fluid and milk by lectin-immunoassay

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The hinge region of IgAl contains five short *O*-linked oligosaccharide side chains which may be sialylated: neutral Galβ3GalNac saccharides were detected on IgAl from human myeloma, mono- and disialylated forms have been shown in case of normal serum IgAl. Recently described lectin, jacalin can discriminate between the two IgA subclasses, because it reacts only with *O*-linked galactosyl (β-1,3) N-acetylgalactosamine. This stucture is also recognized by PNA but only in the absence of sialyc acid. We have developed an immunoassay method using these lectins to investigate the *O*-glycosylation of IgAl present in serum, synovial fluid and human milk. The total IgAl contents and the lectin binding capacity of samples have been measured in parallel using monoclonal antibody based sandwich or lectin immunoassay respectively. By this method no significant difference in jacalin binding capacity of IgAl either in serum or in synovial fluid was found between rheumatoid arthritis (RA) and non RA patients. In contrast with the incapacity of serum IgAl to bind with PNA, IgAl in synovial fluids showed detectable binding. Secretory IgAl in milk bound strikingly high amount of PNA. These suggest that structure DGalβl-3DGalNAc lacking sialic acid occurs on IgAl in synovial fluids and especially in secretory IgAl.

1.2

The purification of alpha-1-acid glycoprotein from normal and rheumatoid plasma without structural degradation

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Alpha-1-acid glycoprotein (AGP) or orosomucoid is a major serum glycoprotein, of unknown physiological function, which is classified as one of the positive acute phase reactants since its plasma concentration becomes elevated two- to five-fold in certain disease states. Additionally, the proportions and identities of the five asparagine-linked complex oligosaccharide chains are altered during several physiological and pathological conditions, this may be functionally significant. The key to studying the structural heterogeneity of AGP is to develop a procedure that will isolate AGP without structural degradation. We have developed a method for the purification of AGP, using procedures unlikely to damage the glycoprotein structure, which was utilised to isolate AGP from samples of normal and rheumatoid plasma. The effectiveness of the purification procedure was examined by enzymatically deglycosylating each sample of AGP and separating the released oligosaccharides by chromatography on a pellicular high-performance anion-exchange (HPAE) resin at pH I3. The analytical profile for normal AGP was consistent with those previously reported thus indicating that the purification procedure did not denature the oligosaccharide chains of AGP. Additionally, there was a noticeable difference between the profiles for AGP from normal and rheumatoid plasma.

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1.3

Isolation of a glycoprotein bearing the H-type I carbohydrate antigen from the murine uterine epithelium

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The H-type I histo-blood group antigen (Fucα1-2Gal-βl-3GlcNAcβl-) has been identified on the murine uterine epithelium using monoclonal antibodies. Previous work has suggested that this carbohydrate antigen functions in the attachment of the embryo at implantation. Further characterization of this antigen would provide a valuable insight to the mechanism of carbohydrate mediated embryo implantation. In this study we have isolated the major glycoprotein from the uterine epithelium which carries the H-type I antigen. It appears to be a trypsin insensitive, monomeric transmembrane glycoprotein of 120-l30 kDa. The glycoprotein can be solubilised from the epithelial membrane by non-ionic detergent or can be released from epithelial cells by sonication. N-glycanase digestion suggests that it contains predominantly *O*-linked carbohydrate chains, some of which carry the H-type I antigen. The molecule can be detected on Western blots by both H-type I specific monoclonal antibody and the fucose specific lectin UEA I. As a tool for further study, we are currently developing monoclonal antibodies to the glycoprotein and its deglycosylated protein core.

1.4

Confirmation of heterogeneity of normal canine ocular mucins

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The mucins of the ocular surface have specialised biochemical, physiological, and rheological requirements due to their multiple roles in ocular defence; the constitution of the pre-ocular tear film; and specific surface chemical interactions with other tear-film components. A detailed analysis of ocular mucins has not previously been presented. Our earlier work [1] has shown differential staining profiles on density gradients using lectins and anti-mucin antibodies, suggesting het-

erogeneity of canine ocular mucins. We now describe a detailed examination of ocular aspirates from dogs, using established methods of mucin purification and analysis. Mucus was collected by suction from the ocular surface of 10 normal male dogs (~12 months old). The pooled sample was dispersed in 4M Guanidine HCl/PBS containing several proteinase inhibitors. Density gradient centrifugation (DGC) was undertaken in CsCl over the range ~1.2-1.7g/ml. Seven pools across this range were collected. Each pool was subjected to gel filtration on Sepharose 4B columns. 'Excluded' fractions were desalted, reduced and alkylated, and electrophoresed on agarose gels. Vacuum blots were probed with lectins and antibodies. Staining profiles of density gradients, and banding patterns on vacuum blots indicate at least two separate types of ocular mucin, each containing distinct subunits. These results show that ocular mucus contains at least 2 distinct mucins each with unique subunits. Evidence for this conclusion is provided by separation of the native mucins on DGC and by resolution of separate subunits on agarose gel electrophoresis. This is the first definitive demonstration of ocular mucin heterogeneity using these methods, and tends to confirm previous suggestions of multiple secretory pathways for ocular surface mucins.

1. S.D. Carrington *et al, Bio Chem Soc Trans* 1993; *21*: 484S.
[Supported by the Wellcome Trust.]

1.5

Comparison of different methods of analysis of immunoglobulin G associated oligosaccharides in rheumatoid arthritis and healthy individuals

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Studies using lectin binding analysis have shown a higher content of agalactosylated oligosaccharides associated with serum IgG in patients with rheumatoid arthritis (RA) compared with healthy individuals. We have directly compared binding to the lectins *Ricinus communis* agglutinin I (RCA I) and *Bandeiraea simplicifolia* II (BS II) with densitometric data obtained by fluorophore assisted carbohydrate electrophoresis to analyse the oligosaccharides associated with IgG from 27 serum samples from 15 patients with RA and 21 serum samples from 21 healthy individuals. IgG was purified from sera by ion-exchange chromatography. The amount of terminal galactose and *N*-acetylglucosamine was determined using specific lectin binding. *N*-linked oligosaccharides were released by incubation with peptide-*N*-glycosidase F, labelled with 8-aminonaphthalene-1,3,6-trisulphonic acid and electrophoretically separated. Densitometric analysis of the bands and expression of the pixel score of each individual band as a percentage of the total score for each lane (relative intensity) was used to compare results. The relative intensity of band 1 from the patients with RA showed a strong positive correlation with RCA I binding ($P<0.001$) and a negative correlation with BS II binding ($P<0.01$). The relative intensity of band 4 from the patients with RA showed a strong negative correlation with RCA I binding ($P<0.001$) and a positive correlation with BS II binding ($P<0.01$). In contrast, there was no correlation between the relative intensities of the same bands with either ricin or BS II binding in the healthy individuals. The correlation between the results from lectin binding and the densitometric analysis of certain bands of labelled oligosaccharides released from serum IgG of patients with rheumatoid arthritis implies that these different techniques are measuring the same phenomenon in these patients. This does not appear to be the case in healthy individuals and may reflect disease specific oligosaccharide changes.

1.6

Characterisation of immunoglobulin G associated oligosaccharide profiles in rheumatoid arthritis, psoriatic arthritis and ankylosing spondylitis using fluorophore assisted carbohydrate electrophoresis

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Studies using hydrazinolysis followed by exoglycosidase digestion and lectin binding analysis have shown a higher content of agalactosylated oligosaccharides associated with serum IgG in patients with rheumatoid arthritis (RA) compared to healthy individuals. We have applied the new technique of fluorophore assisted carbohydrate electrophoresis to analyse the oligosaccharides associated with IgG from 21 serum samples from patients with RA, 10 serum samples from patients with active psoriatic arthritis (PsA), 10 serum samples from patients with active ankylosing spondylitis (AS) and 23 serum samples from healthy individuals. IgG was purified from serum samples by ion-exchange chromatography. *N*-linked oligosaccharides were released by incubation with peptide-*N*-glycosidase F and labelled with 8-aminonaphthalene-1,3,6-trisulphonic acid. The labelled oligosaccharides were electrophoretically separated and the gels photographed. Densitometric analysis of the bands and expression of the pixel score of each individual band as a percentage of the total score for all the oligosaccharide bands in each lane (relative intensity) was used to analyse the data. The same six bands were seen in all subjects. A significant decrease in relative intensity of the least electrophoretically mobile band, band 1 ($p<0.001$), with a corresponding significant increase in relative intensity of a more mobile band, band 4 ($P<0.001$) was seen in RA and PsA patients when compared with healthy controls. The relative intensity of band 1 was less than 2 s.d. below the mean of the healthy individuals in 13 of 21 patients with RA (62%) and 9 of 10 PsA patients (90%). The relative intensity of band 4 was greater than the 2 s.d. above the mean of the healthy individuals in 12 of 21 patients with RA (57%) and 10 of 10 PsA patients (100%). There was a significant increase in the relative intensity of band 4 ($p<0.001$), but no significant difference in the relative intensity of band 1 in the AS patients compared to healthy controls. Band 1 comigrates with an asialo-, agalacto-, di-antennary oligosaccharide and band 4 co-migrates with an asialo-, digalacto-, di-antennary oligosaccharide, both of which are found on human IgG. In conclusion, fluorophore assisted carbohydrate electrophoresis is a useful and efficient tool for the analysis of IgG associated oligosaccharide changes and may be of specific use as a diagnostic tool in early synovitis and the differentiation of arthritic conditions.

1.7

Mass spectrometry: recent glycobiological applications

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High field magnetic sector double focussing mass spectrometry has played a major role in biopolymer analysis in the past 10-15 years and high field instruments fitted with Fast Atom Bombardment (FAB) sources have solved a very wide range of glycoprotein structural problems. More recently, a powerful new method for analysing intact macromolecules, Electrospray Mass Spectrometry (ES-MS), is being successfully applied to glycopeptide and glycoprotein analysis. In this paper we review the types of glycobiological structural problems amenable to MS methodology and highlight the most effective strategies for studying a wide range of glycoconjugates. FAB-MS and ES-MS are powerful tools for characterising diverse structural features in glycoconjugates, including: (1) the degree of heterogeneity and type of glycosylation e.g. *N*- and/or *O*-linked; high mannose, hybrid, complex etc; (2) sites of glycosylation; (3) branching patterns; (4) the number and lengths of antennae and the patterns of substitution with fucose, sialic acids or other capping groups such as sulphate, phosphate, fatty acyl etc; (5) the sequences of all non-reducing structures irrespective of their overall size or the amount of heterogeneity; (6) the presence of *N*-acetyllactosaminoglycan chains and determination of the number of repeats; (7) the fidelity of the carbohydrate structures of recombinat glycoproteins; (8) abnormalities resulting from defects in biosynthesis; (9) changes in glycosylation of mucins and other glycoconjugates in transformed cells; (10) the degree of modification of nuclear and cytoplasmic proteins with O-GlcNAc and the locations of O-GlcNAc attachment. The strategies that are most effective for solving structural problems, particularly those involving the analysis of highly complex mixtures, will be illustrated with examples from our recent work on glycopolymers of biomedical importance including highly immonogenic glycoproteins of parasitic helminths and the lipoarabinomannans of mycobacteria.

We are grateul to the MRC, BBSRC and Wellcome Trust for financial support.

SESSION 2: The Glycosyltransferases

Murine β1,4-galactosyltransferase: analysis of the regulation of a gene that serves both a housekeeping and cell-specific function

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β1,4-galactosyltransferase (β4-GT) is a trans-Golgi resident type II membrane-bound enzyme that performs both a housekeeping function (glycan biosynthesis) and a cell-specific function (lactose biosynthesis). The β4-GT gene specifies two mRNAs of 3.9 and 4.1 kb in somatic cells as a consequence of initiation at two different start sites, located on exon one, and separated by 200 bp. Because the respective transcriptional start sites are positioned either upstream of the first of two in-frame ATGs (4.1 kb) or between the two in-frame ATGs (3.9 kb), translation of each mRNA results in the synthesis of two structurally related, functionally equivalent, trans-Golgi resident protein isoforms that differ only in the length of their respective NH_2-terminal cytoplasmic domains. Somatic cells and tissues, including the virgin mammary gland, use predominantly the 4.1 kb start site. The only exception to this general pattern is found in the mid- to late pregnant and lactating mammary gland where the 3.9 kb start site is preferentially used. This switch to the predominant use of the 3.9 kb transcriptional start site is coincident with the cellular requirement for increased β4-GT enzymatic levels (about 10-fold) required for lactose biosynthesis. These observations, combined with a promoter deletion analysis using β 4-GT/CAT hybrid constructs, have suggested a model of transcriptional regulation in which the distal region functions as a housekeeping promoter while the proximal region functions as a mammary cell-specific promoter.

During murine male germ cell development, there is an additional level of transcriptional regulation of the β4-GT gene that differs significantly from that observed in somatic cells. In spermatogonia, only the 4.1 kb transcript can be detected and it is identical in structure to the 4.1 kb mRNA found in somatic cells. As spermatogonia enter meiosis and subsequently develop into pachytene spermatocytes, β4-GT gene expression is reduced to barely detectable levels. Continued differentiation to haploid round spermatids is coincident with renewed expression to levels comparable to those observed in spermatogonia. However, the 4.1 kb transcript is replaced by two germ cell specific transcripts of 2.9 and 3.1 kb, which have the same open reading frame and utilize either one of two alternative polyadenylation signals embedded within the long 3′-UT region of the somatic transcripts. Both germ cell specific β4-GT transcripts have an additional 5′-UT sequence of 560 nt, that is located immediately upstream and contiguous with the start site defined for the 4.1 kb transcript. These observations indicate that a male germ cell specific promoter regulates expression of the β4-GT gene in the later stages of murine spermatogenesis. We have isolated a genomic fragment that spans the germ cell specific start site, which is sufficient to direct expression of the reporter gene β-galactosidase, exclusively to the pachytene spermatocytes and round spermatids of transgenic mice. This pattern of cell-type specific expression is comparable to that observed for the endogenous β4-GT gene.

1. Harduin-Lepers A, *et al, J Biol Chem* 1993; *268*: 14348-14359.
2. Shaper NL, *et al, J Biol Chem* 1994; in press.

Novel glycosylation routes for glycoproteins: the lacdiNac pathway

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In an increasing number of instances (sub)terminal GalNAc β1→4GlcNAc (*N,N′*-**di**acetyllactos**di**amine, lac**di**NAc) units rather than the more common Galβ1→4GlcNAc (*N*-acetyllactosamine, lacNAc) units are found on the *N*- and *O*-linked oligosaccharide chains of glycoproteins. The synthesis of this unit is controlled by an UDP-GalNAc:GlcNAcβ-Rβ4-*N*-acetylgalactosaminyltransferase (β4-GalNAcT) that has been demonstrated in the mollusc *Lymnaea stagnalis* and in different stages of the avian schistosome *Trichobilharzia ocellata.* This enzyme can be distinguished from all other β4-GalNAcTs described to date including the one of pituitary gland that is involved in the specific glycosylation of the pituitary glycohormones. In many enzymatic properties the β4-GalNAcT resembles mammalian β4-galactosyltransferase (β4-GalT), which controls the synthesis of lacNAc units. Because of this similarity it is proposed that in cells that express both these enzymes there is competition for common substrate sites; the expression levels of each of these enzymes determine whether lacNAc- or lac**di**NAc-type chains will prevail on the glycoprotein products.

The resemblance of β4-GalNAcT and β4-GalT prompted us to isolate DNA sequences from *L. stagnalis* using a cDNA coding for β4-GalT as a probe. Two different but related cDNAs were isolated from the albumen gland and the prostate gland, respectively. In these cDNAs several regions were found that showed considerable similarity with corresponding exons of the murine β4-GalT genome. The amino acid sequence deducted from the prostrate gland cDNA represented a protein that in all respects showed a domain topology typical for a glycosyltransferase (GT). It is expected that one of these cDNAs encodes a β4-GalNAcT that is involved in the synthesis of lac**di**NAc-based structures.

Further processing of lac**di**NAc chains shows analogy with that of lacNAc based chains. *e.g.* Galβ1→4GlcNAc specific α6-sialytransferases can also act on lac**di**NAc to form NeuAc α2→6GalNAcβ1→4GlcNAc and human milk α3(4)-fucosyltransferase can act on this disaccharide to yield the lac**di**NAc analog of the Lewis^x structure (GalNAcβ1 →4[Fucα1→3]GlcNAc). However, GT catalyzed additions of sugars to C-3 of the GalNAc residue in lac**di**NAc, that readily occur to C-3 of Gal residues in lacNAc, proceed at very low rates if at all. This suggests that polylactos**di**minoglycan [(GalNAcβ1→4GlcNAcβ1→3)$_n$] sequences will not generally occur in nature.

The genetic regulation of sialyl-Le^x expression in haemopoietic cells

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The carbohydrate determinant sialyl-Le^x (NeuAcα2-3Galβ 1-4[Fuc(αl-3]GlcNAc) occurs as a cell surface structure on mature neutrophils and serves as a ligand for the adhesion molecule, E-selectin. Binding of these molecules is believed to play an important role in the low level adherence of neutrophils to endothelium ("rolling") that takes place in the first stages of acute inflammtion [1]. Expression of sialyl-Le^x is very much reduced on immature myeloid cells , although these cells strongly express α1,3-fucosyltransferase activity and the Le^x, (Ga1β1-4[Fucαl-3]GlcNAc) determinant [2]. The level of fucosyltransferase activity drops sharply when the promyelocytic cell line, HL60, is induced to differentiate along the granulocytic pathway but the expression of sialyl-Le^x is increased [2]. Use of [^{32}]P-labelled cDNA probes for four α1,3-fucosyltransferase genes, Fuc-TIII, -TIV, -TV and -TVI, that have been cloned [3] showed that Fuc-TIV expression falls on differentiation of HL60 cells and is very weakly expressed in the mRNA of mature neutrophils. Northern analysis of mRNA from either undifferentiated or differentiated HL60 cells with the probes for Fuc-TIII, -TV or -TVI did not show any significant signals. Biochemical studies on the α1,3-fucosyltransferases indicated that the enzymes utilising sialylated glycoprotein acceptors are different from those utilising non-sialylated acceptors in both differentiated and undifferentiated HL60 cells. These results are consistent with the suggestion that another recently cloned gene, designated Fuc-TVII [4], that, *in vitro*, acts only on sialylated acceptors, may be the gene responsible for the biosynthesis of sialyl-Le^x on neutrophils.

This work was supported by the Wellcome Trust

1. Brandley B.K, *et al. Glycobiology* 1993; *3*: 633-639.
2. Skacel P.O, *et al. Blood* 1991; *78*: 1452-1460.
3. Lowe J.B, *Cell Biology* 1991; *2*: 289-307.
4. Natsuka *et al. J Biol Chem* 1994; *269*: 16789-16794.

Molecular analysis of cell surface β1,4-galactosyltransferase expression and function

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β1,4-Galactosyltransferase (GalTase) is unusual among the glycosyltransferases in that it is found in two subcellular compartments where it performs two distinct functions. In the *trans*-Golgi complex, GalTase participates in oligosaccharide biosynthesis, as do the other glycosyltransferases. On the cell surface, however, it associates with the cytoskeleton and functions as a receptor for extracellular oligosaccharide ligands. Although much is known regarding GalTase function in these two compartments, little is known about how it is targeted to these different sites. Cloning the GalTase gene products has identified certain features of the protein that appear to be critical for its expression on the cell surface or retention within the Golgi complex. Studies of cells expressing various GalTase variants, GalTaseCAT reporter proteins, and immunofluorescence confocal microscopy suggest a cytoplasmic sequence unique to one GalTase isoform is responsible for targeting a portion of this protein to the plasma membrane, enabling it to function as a cell adhesion molecule.

2.1

Description of a promoter region supporting transcription of a novel human β-galactoside α2,6-sialyltransferase transcript in HepG2 cells

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β-galactoside α2,6-sialyltransferase (ST) is localized in the Golgi apparatus where it catalyzes the addition of sialic acid to the termini of oligosaccharides on glycoproteins and glycolipids. The enzyme is postulated to be an atypical acute phase reactant, in that a proteolytic fragment harbouring the enzymatic activity, is released to serum during the hepatic acute phase response. In humans, two transcripts differing only in their 5′ untranslated regions, have previously been reported. One of these transcripts is expressed in a number of cell types whereas the other is restricted to mature B-cells. We have previously shown by RNase protection analysis that neither of these transcripts could explain the expression of ST in the hepatoma cell line HepG2. Here we demonstrate the existence of a third human ST transcript in HepG2. This transcript has a shorter 5′ untranslated region than the others, and is read from a promoter region which harbours binding sites for several acute phase inducible and liver-specific transcription factors. This indicates that this transcript may be responsible for the increased expression of ST in liver cells during the acute phase response. Studies on the regulation of this promoter will be presented.

2.2

Recombinant expression and characterisation of an α3 fucosyltransferase

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The interaction of the carbohydrate antigen sialyl Lewis X (sLe^x) with its endothelial ligand, E-selectin, is critical for the adhesion of circulating peripheral blood neutrophils to activated endothelium and their subsequent extravasation from the bloodstream. Despite this the identity of the α3 fucosyltransferase responsible for the biosynthesis of neutrophil sLe^x is unclear. We have cloned FucTVI from human blood neutrophils by RT-PCR and have transiently expressed it in both COS-1 cells and a baculovirus/Sf-9 expression system. Acceptor specificity studies of the recombinant enzyme confirmed that the enzyme was an α3 fucosyltransferase capable of transferring fucose to both sialylated and non-sialylated type II precursors, but not to type I acceptors or lactose. Enzyme activity was optimal in the presence of manganese and at slightly alkaline pH. The α3 fucosyltransferase was sensitive to the sulphydryl reagent *N*-ethylmaleimide, however inhibition could be blocked by the inclusion of GDP-fucose. The use of other amino acid selective reagents has also iden-

tified histidine and tryptophan residues, in addition to cysteine, as being essential for $\alpha 3$ fucosyltransferase activity. Further characterisation of FucTVI, in addition to other fucosyltranferases, is ongoing.

2.3

$\alpha 2,6$ sialyltransferase and $\beta 1,4$ galactosyltransferase activity in CD19$^+$ peripheral blood lymphocytes

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It is well established that the biological activity of peripheral blood B cell lymphocytic $\beta 1,4$ galactosyltransferase (GTase) is reduced in rheumatoid arthritis (RA). In order to determine whether this reduction is a unique phenomenon or part of a wider change in the *N*-linking golgi glycosyltransferase pathway, we have assayed the activities of the two terminal transgolgi glycosyltransferase, namely $\alpha 2,6$ sialyltransferase (STase) and 1,4GTase in CD19$^+$ peripheral blood B-cells. GTase was assayed using ovalbumin as a glycoprotein acceptor for the transfer of ^{3}H-galactose, STase using asialotransferrin for the transfer of ^{14}C-sialic acid. CD19+ cells were isolated from the peripheral blood mononuclear cell isolates of 10 RA patients and 9 age and sex matched healthy controls, using anti-CD19 Dynabeads. The GTase activity was significantly reduced in the RA group 8,673 + 3,536 cpm/10^6 cells/hour compared to 15,751 ± 11,175 cpm/10^6 cells/hour for the controls ($P<0.05$). STase activity was also significantly reduced in the RA group 10,472 + 4,514 cpm/10^6 cells/hour compared to 16,464 ± 9,197 cpm/10^6 cells/hour for the controls ($p<0.05$). Further analysis of the data revealed a significant positive correlation between these two glycosylation enzymes, GTase and STase in both the RA and healthy control populations ($r=0.911$, $P<0.0001$). Interestingly the concomitant reduction in STase does not appear to correlate with the degree of IgG sialylation, as demonstrated by the results of our preliminary study showing differential IgG sialic acid and galactose changes in RA (decrease in galactose whilst sialic acid remained constant). It is possible that, since only 20% of the IgG oligosaccharides are sialylated [1], that a down-regulation of STase activity would not manifest itself, since it may still be present at levels sufficient to fulfil the modest sialylation requirements of IgG. The crucial question now, is what controls the changes in the activities of both of these enzymes?

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2.4

$\beta 1,4$-Galactosyltransterase gene expression in rheumatoid arthritis

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Serum IgG is under-galactosylated in both rheumatoid arthritis (RA) and in animal models of this disease, and is associated with reduced β-1,4-galactosyltransferase (β-1,4-GalTase) activity levels. In order to elucidate the mechanism of reduction in enzyme activity, we have used RNase protection assays to quantitate β-1,4-GalTase mRNA expression in both RA and animal models. We have previously observed that the MRL-*lpr/lpr* murine model of human RA shows a 60% reduction in β-1,4-GalTase mRNA in splenic lymphocytes compared to splenic lymphocytes in the normally glycosylated CBA/Ca strain [1]. In preliminary studies, peripheral blood mononuclear cells (partially depleted of monocytes by plastic adherence) showed comparable levels of β-1,4-GalTase mRNA in six patients with RA compared to six normal controls when using β-tubulin as a control probe to standardize RNA levels in each lane. Ribosomal RNA is more stably expressed than other control housekeeping genes commonly used [2]. Using 18S rRNA as a control probe and in the absence of a plastic adhesion step, we observed reduced levels of β-1,4-GalTase mRNA in eight patients with RA compared to eight normal controls ($P<0.005$). Experiments are in progress to determine whether or not the control probe and/or variation in the cell population contributed to these different results. These studies are important in establishing the role of reduced β-1,4-GalTase gene expression in the control of IgG galactosylation in RA.

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2. Finnegan M.C., *et al. Leuk-Lymphoma* 1993; *10*: 387-393.

2.5

Changes in β-1,4 galactosyl and α-2,6 sialyltransferase of human B-cell clones induced by *mycoplasma fermentans*, which is a possible factor in the aetiology of rheumatoid arthritis

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M. fermentans is a human commensal or pathogen, which may play a possible role in the pathogenesis of rheumatoid arthritis (RA). Evidence in support of this includes isolation of *M. fermentans* from the joints of patients with RA, reports of cell-mediated immune response to this organism in these patients and finally, the use of mycoplasma infection as a model of chronic joint inflammation. To investigate the relevance of these observations, we have examined the affect of *M. fermentans* infection on various RA associated glycosylation parameters, in cloned Epstein-Barr virus (EBV) transformed B cells derived from both normal healthy ($n=3$) and RA ($n=1$) individuals. *M. fermentans* infection caused an overall significant reduction in both, β-1,4 galactosyltransferase (GTase) and α-2,6 sialyltransferase (STase) activity, in the clones examined ($n=10$, $P<0.05$). Further analysis of the data revealed that, in contrast to the reduction (-70%) in GTase activity, which was confined to the IgG ($n=6$) secreting clones, the STase reduction (-30%) was limited to the IgM secreting B cells ($n=4$), and the IgG secreting B cells in the normals only. Further studies to investigate the relevance of these *M. fermentans* induced immunoglobulin-class specific glycosyltransferase changes to the pathogenesis of RA are now in progress.

2.6

Measurement of lymphocytic β1,4-galactosyltransferase activities in the presence of a neoglycoprotein

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β1,4-Galactosyltransferase (β1,4-GalTase) catalyses the transfer of galactose from UDP-galactose to the non-reducing terminal end of *N*-acetylglucosamine (GlcNAc) in the presence of Mn^{2+}. Two different acceptor substrates with terminal GlcNAc residues, ovalbumin and a synthetic neoglycoprotein, *N*-acetyl-β-D-Glucosamine-phenylisothiocyanate-BSA (GlcNAc-pITC-BSA), were compared in two separate assay systems for the measurement of lymphocytic β1,4-GalTase. The commonly used radiochemical assay measures the transfer of radio-labelled galactose (Gal) from UDP-^{3}H Gal to a suitable acceptor containing a non-reducing terminal GlcNAc. This was compared with an ELISA-based assay whereby the acceptor substrate is coated onto micro-titre well ELISA plates and any transfer of Gal from UDP-Gal detected with biotinylated *Ricinus communis I* lectin which specifically binds to terminal Galβ1-4GlcNAc structures. These assays were used to detect lymphocytic β1,4-GalTase in tonsils and in peripheral blood from rheumatoid arthritis (RA) patients and controls. The sensitivities of both assays were enhanced using the GlcNAc-pITC-BSA as the acceptor substrate. Whilst the radiochemical assay measured β1,4-GalTase activity from 15μU (lactose synthase units) compared to the ELISA which measured from 100μU, both assays proved suitable for the measurement of lymphocytic β1,4-GalTase. Indeed, the established reduction of β1,4-GalTase activity in B cells from RA patients, $n=8$, when compared to controls, $n=10$, were measured using the ELISA method and proved to be very reproducible (sample c.v. <5%) and rapid to perform. In summary, the ELISA method using the GlcNAc-pITC-BSA acceptor substrate is preferred over the radiochemical assay for the measurement of β1,4-GalTase on the basis of cost and practicability.

2.7

Galactosyltansferase isoenzymes in rheumatoid arthritis and healthy individuals

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β-1, 4 galactosyltransferase(GTase), a soluble or membrane bound enzyme transfers galactose from UDP-galactose to terminal *N*-acetylglucosamine residues during oligosaccharide modification. The activity of this enzyme is reduced in both B and T cells of rheumatoid arthritis (RA) patients and this reduction may be responsible for the changes associated with complex oligosaccharides on immunoglobulin G (IgG) in RA and a restricted group of other inflammatory states. Different GTase isoenzymes may be responsible for the incorporation of galactose in different glycoproteins. This was suggested by studies in which B cell GTase activity was compared using a variety of different acceptor molecules, where differential incorporation of galactose onto the acceptors was demonstrated. The aim of this study was to determine whether there are RA specific GTase isoenzyme profiles.

• **Soluble GTase.** Serum from both active RA patients and healthy individuals were fractionated by preparative liquid phase isoelectric focussing between pH 3-10. GTase activity peaks thought to represent isoenzymes were defined by subtracting the mean plus 1 standard deviation from each of all the fractions obtained. In healthy controls, peak GTase activity occurs between pH 4.0-5.0 whereas in RA there is a significant absence of enzyme activity below pH 5.0 ($P=<0.05$), representing a shift in GTase isoenzyme activity to above pH 5.0. • **Membrane bound GTase** Peripheral B cell (CDI9) extracts from 10 RA and 9 healthy individuals were run on pH 3-10 gradient isoelectric focussing agarose gels. When focussed, gel slices were cut out and assayed for enzyme activity. Both RA and control preparations had peaks of activity above pH 8.2. Four of nine control preparations had additional enzyme activity in the pH range 7.5-8.2, whilst only 1/10 RA preparations showed such activity. In contrast to the control preparations, the remaining RA enzyme activity was spread throughout the pH range. The results indicate the likelihood of differential isoenzyme expression of both B cell membrane bound as well as soluble serum forms of GTase, which may change in RA.

2.8

Isolation of glycosyltransferase cDNAs from *Lymnaea stagnalis*

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Glycosyltransferases form a functional family of membrane bound enzymes that are involved in the coordinate biosynthesis of oligosaccharide chains on proteins and lipids. The enzyme GlcNAcRβ-Rβ4-galactosyltransferase (β1,4GalT), controls the synthesis of the Gal, β1-4GlcNAc-R (LacNAc) unit, commonly found on the complex-type carbohydrate chains of mammalian glycoconjugates. Evidence is accumulating that in many instances, particularly in invertebrates, a GalNAc is incorporated in the place of a Gal, resulting in a so called LacdiNAc-type structure (1). In the pond snail *L. stagnalis* the enzyme responsible for the synthesis of this type of structure, UDP-GalNAc: GlcNAc-Rβ1→ 4-N-Acetylgalactosaminyl-transferase (β1,4 GalNAcT), has been demonstrated (2), whereas this organism does not contain β1,4GalT. Because of the analogy of the reactions catalyzed by β1,4GalT and β1,4GalNAcT we anticipated that a molecular homology would exist between these enzymes, that could be exploited to isolate cDNA(s), related to the β1,4GalT cDNA. Using bovine β1,4GalT cDNA (3) as a probe, we isolated two different cDNAs from *L. stagnalis,* with sequence identity to the β1,4GalT cDNA. Expression studies are in progress to determine the enzymatic properties encoded by these cDNAs.

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SESSION 3: Oligosaccharides and Recognition

Sialomucins in inflammation and hematopoiesis

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Mucin-like glycoproteins are expressed in a diverse set of tissues. However, the functions of most of these mucins are currently unknown. One of the most interesting possibilities is that these highly glycosylated molecules perform functions that go beyond the currently accepted roles as lubricants and anti-pathogens. We have investigated the functions of two sialomucins, GlyCAM 1 and CD34. in inflammation and hematopoiesis. Both of these mucins can function as ligands for L selectin by presenting unique carbohydrates to the lectin domain of this leukocyte adhesion molecule. GlyCAM 1 is a soluble ligand for L selectin, and the production on knockout mice has revealed that this mucin appears to function as a regulator of lymphocyte trafficking during inflammation. The expression of this mucin appears to be unregulated on the endothelium during inflammation, suggesting that it might function to dampen the influx of leukocytes. Like GlyCAM 1, CD 34 can also function as a ligand for L selectin-mediated adhesion. This mucin is expressed at a diversity of endothelial sites in the adult and in the embryo. In addition, it is also expressed on hematopoietic progenitors in both the adult and in the embryo. The expression of CD34 on hematopoietic cells of the yolk sac has led to the finding that this fraction of cells contains the hematopoietic progenitors, a result that is similar to what has been found in later stages of hematopoiesis. This suggests that CD34 may play a critical role in hematopoiesis, and knockout studies in cell culture support this hypothesis. Thus, embryonic stem cells that have had both alleles of CD34 knocked out have defects in the production of hematopoietic cells when they are allowed to develop into embryoid bodies in culture. We are also currently assessing the role of CD34 *in vivo* in knockout mice. In summary, it appears that both GlyCAM 1 and CD34 play important roles in inflammation and hematopiesis, consistent with the possibility that sialomucins perform a complex variety of functions.

A human T cell receptor recognises O-linked sugars from the hinge region of human IgA1 and IgD

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A receptor which binds secretory IgA (sIgA) is expressed on human T-cells from patients with systemic lupus erythrematosus, rheumatoid arthritis, Behcet's syndrome and IgA nephropathy and on normal T-cells following PHA stimulation. The specificity of this receptor was initially probed with a panel of normal serum immunoglobulins in competitive inhibition assays with sIgA using two colour immunofluorescence. While the receptor showed the strongest affinity for IgA1 ($IC_{50}10^{-5}$M), IgD which has a similarly glycosylated hinge region to IgA1, also bound ($IC_{50}10^{-5}$M). IgA2, which lacks the *O*-glycosylated hinge region, did not significantly inhibit the binding at these concentrations suggesting that the IgA determinants for this receptor might be the oligosaccharides present in the hinge region of IgA1. IgA1 has up to 10 *O*-linked oligosaccharides and four *N*-linked oligosaccharides per molecule. In order to probe the role of the *O*-linked hinge sugars in the binding event, a sugar library was prepared from IgA1 by a procedure designed to release *O*-linked oligosaccharides preferentially, and to retain them in the natural closed ring formation. The sugars were released by hydrazinolysis at 65°C and the resulting oligosaccharide library analysed by high voltage paper electrophoresis and P4 gel permeation chromatography. Competitive inhibition studies demonstrated that both the library and the individual *O*-linked sugars associated with IgA1 were implicated in the binding of IgA1 to this receptor (IC_{50} between $1x10^{-5}$M and $6x10^{-5}$M). Within this range the individual sugars showed small differences in their affinity for the receptor in the following order:
Galβ3GalNAc=NeuNAc2α3(6)Galβ3Ga1NAc>NeuNAc2α 3(6)Galβ3[NeuNAc2α6]GalNAc$\geq$GalNAc

Carbohydrate recognition by the selectins and other C-type lectins

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The selectins are members of a family of multidomain proteins characterised by the presence of one or more Ca^{2+}-dependent carbohydrate-recognition domains. The three selectins E, P and L have different topologies but they are all involved in the initial adhesion events - the contact between circulating leukocytes and endothelium - in the inflammatory cascade. Other members of the C-type lectin family include serum mannan-binding protein, conglutinin and pulmonary surfactant apoprotein A which are involved in innate immunity. Considerable information is accumulating on carbohydrate recognition by these C-type lectin proteins. Unravelling the combining specificities of members of this family of proteins is important for understanding their various roles in responses to injury and microbial infections, the haematogenous spread of cancer cells and for design of novel antagonists for therapy of disorders of inflammation. This communication will focus on developments in our understanding of oligosaccharide recognition by E and L selectins; these will be discussed within the framework of our knowledge of the various modes of carbohydrate-recognition by C-type lectins. The range of oligosaccharide ligand structures recognised, the requirement for clustering of protein and ligand, and the influence of carrier protein or lipid on oligosaccharide recognition will be highlighted.

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5. Green PJ, *et al.* submitted.

3.1

Heterogeneity of cell surface CD antigens on human leucocytes

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The antigenic determinants on some human leucocyte CD (cluster of differentiation) molecules are oligosaccharides. These include CDw75, a heterogeneous group of related cell surface antigens developmentally regulated during human B-lymphopoiesis, and expressed at high levels on mature *B* lymphocytes [Guy K. & Andrew *J.M., Immunology* 1991, 74:206-214]. CDw75 antigens are also subset-specific markers of T lymphocytes *[ibid]* and blood-group-like antigens on red cells [Guy K. & Green *C. Immunology* 1992, 75: 713-716] . CDw75 and the complex oligosaccharide blood group I may be related in structure [Guy K.,Tippett P. & Ross J .A. *Leucocyte Typing V,* in press]. Many different cell surface proteins, glycoproteins and glycolipids expressed on human leucocytes have been characterised in the CD system at Leucocyte Typing Workshops. Notwithstanding this, microheterogeneity of oligosaccharide structures on CD antigens implies that leucocyte cell surfaces are even more structurally complex than is presently evident. The restricted expression of some carbohydrate CD antigens contrasts with the apparently more widespread cellular distribution of the corresponding glycosyltransferases. Recent findings suggest that high levels of expression of novel mRNA isoforms of alpha-2, *6* sialyltransferase (STase) in human B lymphocytes are differentiation-related and accompany the appearance of CDw75 antigens [Wang X.C.*et al, J Biol Chem* 1993; *268*: 4355-4361]. To investigate the relationship between STase and CDw75 expression in leucocytes, we have prepared monoclonal antibodies against a synthetic peptide corresponding to the predicted amino acid sequence of the enzyme.

3.2

Sialic acid binding properties of sialoadhesin, CD22 and MAG

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Sialoadhesin is a receptor expressed by macrophages in the bone marrow and in some lymphoid tissues which recognises cell-surface sialylated glycoconjugates. Sialoadhesin is a member of the immunoglobulin (Ig) superfamily with 17 Ig-like domains. This receptor has greatest homology to CD22, a B cell-specific adhesion molecule, and to myelin-associated glycoprotein (MAG), an adhesion molecule of oligodendrocytes and Schwann cells which has been implicated in myelination. CD22 also recognises sialylated ligands, however the binding of MAG to sialic acid has not been described. We have investigated the binding properties of these three receptors either expressed in COS cells or as chimaeric proteins containing the Fc portion of human IgG. Like sialoadhesin and CD22, MAG bound erythrocytes in a sialic acid-dependent manner. In experiments using erythrocytes modified to carry only one type of sialylated oligosaccharide, MAG recognised only Neu5Acα 2, 3Galβ1, 3GalNAc, whereas sialoadhesin bound to Neu5Acα 2, 3Galβ1, 3GalNAc or Neu5Acα2, 3Galα1, 4GlcNAc and CD22 bound only Neu5Acα 2, 6Galα 1, 4GlcNAc. When the binding of these receptors to various cell populations was investigated, sialoadhesin and MAG showed greatest binding to neutrophils whereas CD22 bound preferentially to lymphocytes. In addition, MAG was found to bind to isolated neuronal cells in a sialic acid-dependent manner. Therefore, sialoadhesin, CD22 and MAG are structurally related molecules, expressed by highly restricted cell populations, which mediate cellular interactions by binding specific sialylated ligands

3.3

Interaction of sialoadhesin, MAG and CD22 with sialylated ligand

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Sialoadhesin, MAG and CD22 are members of a recently defined family of sialic acid-dependent adhesion molecules within the immunoglobulin superfamily. Sialoadhesin is expressed on murine macrophages in haemopoietic and lymphoid tissues and proposed to interact with leukocytes. It specifically recognises sialylated ligands with terminal Neu5Acα 2→3Galβ1→3GalNac (3-0) or Neu5Acα 2→3Galβ1→3 (4) GlcNAc (3-N) glycans. MAG is found on myelinating oligodendrocytes and Schwann cells and probably mediates contact with neuronal cells. For binding MAG requires 3-0 glycans as ligands. CD22 is a B-cell restricted molecule and believed to interact with lymphocytes by binding to Neu5Acα2→6Galβ1→4GalNac (6-N).

Protein-carbohydrate interactions are often multimeric. Therefore, we investigated the influence of receptor valency on binding of cells or soluble ligands. Fc-chimaeras of the adhesins were complexed in solution with variable concentrations of human anti IgG (Fc) antibodies or immobilised on microtiter plates. Binding to human erythrocytes and GT1b-BSA used as ligands was dependent on a minimum receptor density. Furthermore, in binding experiments with erythrocytes carrying limited densities of ligands a threshold glycan density for stable sialoadhesin binding was observed.

No significant binding of erythrocytes to CHO-cells expressing MAG was observed. However, after sialidase treatment of these cells, erythrocytes adhered strongly. This is evidence that CHO-MAG cells express ligands which are recognized by MAG and thus occupy binding sites for ligands on erythrocytes.

In conclusion, we could show that multivalent receptor-ligand interactions are required to mediate cell adhesion through these proteins. In addition, we supply evidence for *cis* interaction of MAG with ligands on the same cell membrane.

3.4

Slex is not responsible for the interaction of slex positive memory T lymphocytes with E-selectin

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E-selectin is an adhesion molecule which is transiently and exclusively expressed on endothelial cells in response to inflammatory cytokines. In addition, E-selectin participates in the initial interaction of leucocytes with activated endothelial cells. This role of E-selectin in cell adhesion has made it a potential target for modulation of inflammatory processes that for example are occurring in autoimmune diseases like rheumatoid arthritis. The ligand for E-selectin on granulocytes is sialyl Lewis^x (sLex). However, in contrast the molecular nature of the E-selectin ligand on T lymphocytes has not yet been identified. In the present study it is shown by FACS analysis that T lymphocytes stimulated with PHA, IL-2 and TGF-β1 express sLex using the CSLEX1 mab. Furthermore, in a cell adhesion assay these activated T cells of the memory phenotype display specific binding to Chinese hamster ovary (CHO) cells expressing E-selectin whereas no binding is observed to control CHO cells. This adhesion can be blocked with an anti E-selectin mab but not with CSLEXl mab. In the same assay, the interaction of sLex positive U937 cells with the E-selectin transfected CHO cells can be inhibited both with anti-E-selectin and CSLEXl mab's. This implicates that the expression of sLex on activated T lymphocytes is not responsible for the interaction with E-selectin. These results indicate that stimulated T lymphocytes express additional E-selectin ligand(s) with much higher avidity for E-selectin than sLex.

3.5

The regulation of CMP-N-acetylneuraminic acid hydroxylase

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Sialic acids are a group of acidic sugars which occur in the heterooligosaccharide chains of cell surface and secreted glycoconjugates. Their occupation of predominantly exposed positions and their structural heterogeneity enables sialic acids to regulate a number of intercellular recognition processes, including those involved in the immune response and inflammatory reactions. The sialic acid *N*-glycoloylneuraminic acid (Neu5Gc) is of immunological interest, since it can influence the recognition of certain carbohydrate structures by the sialoadhesin from mouse macrophages and the B-cell - associated receptor CD22. Although Neu5Gc is absent from healthy human tissues, certain human tumours possess minute amounts of this sialic acid, associated with antigenic gangliosides called Hanganutziu-Deicher antigens. The regulation of Neu5Gc formation may therefore be of importance in influencing certain immune reactions and in the development of tumours. Neu5Gc is synthesised as its CMP-glycoside by CMP-Neu5Ac hydroxylase, a cytosolic, cytochrome b_5-dependent monooxygenase. Although this enzyme is influential in determining the level of sialylation with Neu5Gc, little is known about the mechanism regulating the activity of the hydroxylase. A polyclonal antibody was raised against the hydroxylase from pig salivary glands and used in a Western blot procedure to quantify the amount of hydroxylase in supernatants of various tissues. A qualitative correlation between the enzyme activity and the amount of immuno-reactive hydroxylase was observed, suggesting that hydroxylase activity is governed by the amount of enzyme protein.

3.6

Oligomannosidic glycan mediated interaction between neural cell adhesion molecules induces signal transduction

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Oligomannosidic glycans *N*-glycosidically linked to the neural cell adhesion molecule L1 mediate association with the neural cell adhesion molecule NCAM. That this association is implicated in neurite outgrowth we could show by adding oligomannosidic glycans to the culture medium of murine early postnatal cerebellar neurons, which disturbed the L1/NCAM interaction resulting in a strongly reduced neurite outgrowth [1]. Based on these observations, signal transduction mechanisms are studied with the aim to elucidate the intracellular responses elicited by this particular interaction, which ultimately influence neurite outgrowth. We present evidence that phosphorylation of L1 is one step of the signalling pathway.

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3.7

Arthritis associated expression of sacolectin-binding receptors in human synovial tissue

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Sarcolectin (SL) is a sialic acid binding endogenous lectin that exerts growth promoting activity on mesenchymal cells. In addition, as an antagonist to interleukin-1 (IL-1), it is involved in immunoregulation. To determine its functional importance the localization of SL-binding receptors in human synovialis was analyzed. Normal knee joint specimens (n=18) as well as inflammatorily altered biopsies (rheumatoid arthritis n=25, activated osteoarthritis n=14) were investigated. Binding patterns of purified (by affinity chromatography) biotinylated SL were compared with the distribution of monoclonal antibodies specific for fibronectin, laminin, tenascin, macrophages (CD 14, MAK 1, M 814, aggregated galactose) and lymphocytes (CD 3, CD 4, CD 20). Histological findings were correlated with clinical and laboratory parameters indicating inflammatory activity. IL-1-levels were determined by enzyme-linked immunosorbent assay. Only 2 out of 18 control specimens revealed faint SL-binding. In contrast, 26 of 39 inflammatory synovial biopsies showed affinity for SL in the extracellular matrix. Staining was restricted to sharply delimited areas surrounding microvasculature, respectively thin layers of synovial tissue bordering the articular lumen. Binding intensity correlated with proof of fibronectin deposition or accumulation of activated macrophages. High IL-1 concentrations in synovial fluid concurred with intense staining reactions. The results suggest that interactions between SL and matrix components may be involved in inflammatory joint diseases. Growth promoting activity may contribute to proliferation of synovial lining cells.

SESSION 4: Glycoforms and Biological Function

Glycosylation of antibody molecules: a small step for structure, a leap for function

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Attachment of a complex oligosaccharide moiety to asparagine 297, of human and mouse IgG, has been shown to be essential for the expression of 'wild type' effector functions. Thus, whilst residual binding activity of aglycosylated IgGs for human FcγRI, mouse FcγRII and Clq have been demonstrated biological activities mediated through human FcγRII and FcγRIII and the C1 component of complement are ablated. Our studies of the nature of the protein/oligosaccharide interactions in this molecule lead us to conclude that the quaternary protein structure of aglycosylated IgG is almost indistinguishable from that of the glycosylated form. Thus, twenty Fc epitopes, probed with mouse monoclonal antibodies in an ELISA assay, are expressed by both forms of IgG3. When the assay was conducted at 56° there is no evidence for loss of epitope expression and at 70° only one epitope was affected; temperatures chosen because structural transitions were detected in native Fc molecule both by Fourier transformed infra-red spectroscopy and micro calorimetry. A limited, localised but significant structural difference between glycosylated and aglycosylated IgG3 was observed by high field NMR spectroscopy. Replacement of an amino acid contact residue for a core sugar residue results in loss of biological activity while no effects were observed when similar replacements of amino acid residues making contacts with outer arm sugars were introduced. We suggest that an optimal conformation for both the protein and the oligosaccharide moieties is an essential requirement for the expression of 'wild type' effector functions.

α_1-Acid glycoprotein: a naturally occurring anti-inflammatory molecule?

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Various glycoforms of α_1-acid glycoprotein (AGP) can be distinguished in human sera. They all contain five N-linked glycans, but they differ in the presence of di-, tri- and/or tetraantennary glycans (type of branching) and in the degree of fucosylation and sialylation. The proportions of the AGP glycoforms are dependent on the (patho)physiological state. For instance, AGP glycoforms with increased diantennary glycan content are transiently induced by acute inflammatory states, whereas the levels of AGP glycoforms containing only tri- and tetraantennary glycans are increased during late pregnancy and estrogen treatment. Both during acute inflammation and in rheumatoid arthritis, a persistent increase in heavily fucosylated AGP glycoforms is apparent. These glycoforms also express the biologically important bloodgroup structure sialyl Lewis^x. All the glycoforms detectable in sera can be secreted by isolated hepatocytes. Cytokines and corticosteroids are involved in the regulation of their biosynthesis. Several *in vitro* studies have shown that AGP can modulate the immune system, e.g., by inhibition of the proliferation of PBL's and by induction of an IL-1-receptor antagonist by PBMC's. Such effects are concentration dependent, reach optima in the physiological range of concentrations, and, as far as determined, involve the carbohydrate portion of the molecule. Especially the inflammation-induced expression of sialyl Lewis^x on AGP is expected to be of great significance in the course of the inflammatory process, because this structure plays a crucial role in a number of biological processes, of which the selectin-mediated interaction between leukocytes and endothelial cells in homing and inflammatory processes are currently most fascinating. Changes in the levels of the various AGP glycoforms, therefore, are expected to be of importance in the function of these molecules in the inflammatory reaction.

The ability of β-galactosidase treated anti-type II collagen monoclonal antibodies to passively transfer arthritis

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Patients with rheumatoid arthritis exhibit increased expression of agalactosyl IgG and a synovial β-cell population skewed towards the synthesis of anti-type II collagen (CII) IgG [1]. It is however unclear whether agalactosyl IgG is a marker for RA or more directly involved in the pathogenesis [2]. Passive transfer studies utilizing single monoclonal antibodies (MAb) with specificity for CII cannot induce clinically overt arthritis [3]. We are now investigating the possible conversion of non-pathogenic MAb's into pathogenic MAb's by altering their glycosylation *in vitro*. Purified anti-CII monoclonals (D3;IgG_{2a}, C2;IgG_{2b} and E8,IgG_1 [3] a gift of R. Holmdahl) were incubated with 6646K, β-galactosidase (β-gal) an enzyme able to digest the normally inaccessible IgG Fc region galactose residues. Following an immunization protocol that primed the T-cells of DBA/1 mice, anti-CII monoclonals (200μg) were given i.v. After 10 days sera levels of agalactosyl IgG [4], anti-chicken CII and bovine CII titres were determined. The results show that contrary to expectations, significant amounts of each monoclonal were still circulating after 10 days. The amount of monoclonals circulating were in order D3>C2>E8 with D3 being essentially uncleared, β-gal treatment made no significant difference to the circulating levels. One mouse receiving the β-gal treated C2 generated a significant anti-bovine CII response (195μg/ml) but did not raise an anti-chicken CII titre, this data suggests the mouse produced a novel anti-CII antibody. The agalactosyl IgG levels of all the mice injected with β-gal treated C2 was considerably elevated. The groups of mice receiving, β-gal treated D3 and E8 antibodies showed elevated acute arthritic scores (within the first 72 hrs) compared to those receiving untreated monoclonals no differences were seen for mice receiving the

F3(control) or C2 monoclonals. The mean arthritic score after 10 days for mice receiving the D3 and E8 monoclonals was the same for both treated and non β-gal treated groups. In contrast the mean arthritic score of mice receiving C2 without β-gal treatment was greater than the C2 β-gal treated group. This data suggests that both glycosylation status and isotype can uniquely influence both acute and chronic arthritis produced by passive transfer of single MAb's with specificity for CII. The possibility that the mechanism of action of agalactosyl IgG may involve complement fixation via the serum lectin mannose binding protein (MBP) is currently being investigated. Early experiments have shown that the C2 and D3 β-gal treated MAb's in contrast to E8 when immobilised to bovine CII coated plates show increased binding to biotinylated-MBP compared to the untreated MAb.

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Immunology of glycosaminoglycans

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Glycosaminoglycans are polymers with backbones of repeating disaccharide units containing a hexosamine and either a hexuronic acid (heparin/heparan sulfate, chondroitin/dermatan sulfate and hyaluronan) or a galactose (keratan sulfate). Three of the four classes of glycosaminoglycans (keratan sulfate, heparin/heparan sulfate and chondroitin/ dermatan sulfate) are extensively modified by the addition of sulfate esters at different locations in their backbone structures. These three classes of glycosaminoglycans are synthesized on a large variety of core proteins to produce macromolecules referred to as proteoglycans, which have manifold functions inside cells, on cell surfaces and in the extracellular matrices surrounding cells [see 1 for reviews].

Variable substitutions and locations of the sulfate esters along native glycosaminoglycan chains, combined with the structure of the carbohydrate backbones, present topographies to immune systems which can be recognized as antigenic. This was initially observed when a majority of the monoclonal antibodies generated in mice immunized with chondroitinase digested cartilage proteoglycans recognized epitopes against highly sulfated regions of keratan sulfate [2]. Subsequently, monoclonal antibodies have been generated against each of the different categories of sulfated glycosaminoglycans. Those that recognize chondroitin/dermatan sulfate will provide the focus of this presentation. These monoclonals can be divided into two subgroups, those which require prior digestion with chondroitinase enzymes to generate their epitopes and those that recognize native epitopes that may be located either in the interior of the chain or at the non-reducing terminus. The former group were generated by immunizing mice with chondroitinase ABC digested cartilage proteoglycans. This enzyme is an eliminase in which glucuronic acid residues in the interior of the chains are converted to 4,5 unsaturated residues, generating unsaturated disaccharides from all the interior disaccharides and leaving a residual unsaturated uronic acid residue on the oligosaccharide stub that provided the original attachment for the chain to the core protein (Figure 1). This 'hapten' structure on the core protein is highly antigenic, and several monoclonal antibodies with specificities for different sulfate substitutions on the adjacent *N*-acetylgalactosamine have been developed using this antigen. Antibody 3B3, which has recently generated widespread interest [3], is one of these antibodies, and it recognizes unsaturated uronic acid residues adjacent to 6-sulfated *N*-acetylgalactosamine. Several antibodies have been described for the second group, but the epitope structure of only one, designated MO-225, has been determined with precision; glucuronic acid residues substituted with 2-sulfate are essential [4]. Antibody 3B3 is unusual in that it belongs to this group as well as the first group since later work [5] showed that it also recognizes native chondroitin sulfate chains from certain cartilage proteoglycan preparations, notably from fetal and osteoarthritic cartilages, but seldom from normal adult articular cartilages. Because the epitope recognized is sensitive to periodate treatment, which would destroy unsubstituted glucuronic acid residues at non-reducing ends of the chains, it was proposed that 3B3 recognizes non-reducing terminal glucuronic acid adjacent to 6-sulfate *N*-acetylgalactosamine in addition to its unsaturated counterpart.

We have now developed highly sensitive methods to determine the structures of the non-reducing termini of chondroitin/dermatan sulfate chains. These include digesting the proteoglycan with chondroitinase, removing the core protein from digestion products and derivatizing the reducing terminal *N*-acetylgalactosamine residues generated by the enzyme with 2-aminopyridine to introduce a fluorescent tag that reports molar proportions of all digestion products. Monosaccharide products (derived from chains with variously substituted *N*-acetylgalactosamine residues at their non-reducing termini) were separated and quantitated on a high resolution ion exchange column (Dionex IonPac AS4A). The small proportion of saturated disaccharides (derived from chains with glucuronic acid at their non-reducing termini) were resolved on the same column after selectively destroying the large proportion of interior unsaturated disaccharide products by mercuric ion treatment. These procedures have been used to map the non-reducing termini of cartilage proteoglycans isolated from fetal and normal adult human cartilages. About 75% of the chains on the fetal proteoglycan were terminated with glucuronic acid adjacent to either 4- or 6-sulfated *N*-acetylgalactosamine in nearly equal amounts, with the remaining 25% substituted with 4-sulfated *N*-acetylgalactosamine. In contrast, about 95% of chains on the adult proteoglycan were terminated in either 4-sulfated or 4,6-disulfated *N*-acetylgalactosamine, with the remaining 5% predominantly glucuronic acid adjacent to a 6-sulfated *N*-acetylgalactosamine. These structures would be consistent with the ability of monoclonal 3B3 to recognize native fetal, but not normal adult cartilage proteoglycans since a much larger proportion of the chains on the former terminate in glucuronic acid adjacent to 6-sulfated *N*-acetylgalactosamine. These procedures combined with immunoaffinity methods to separate glycosaminoglycan chains, or fragments of chains, which contain epitopes from those which do not should facilitate determination of the precise structures recognized by various monoclonal antibodies which recognize glycosaminoglycan epitopes.

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Figure 1. Chondroitinase ABC digestion of chondroitin sulfate backbone structure

R-(glcUA-galNAc)$_n$-glcUA-galNAc-glcUA-gal-gal-xyl-serine

⇓

R+n($_\Delta$UA-galNAc)+$_\Delta$UA-galNAc-glcUA-gal-gal-xyl-seine

where R is the non-reducing terminus, glcUA is glucuronic acid, galNAc is N-acetylgalactosamine, gal-gal-xyl is the galactosyl-galactosyl-xylosyl linkage of the chain to the core protein, and ΔUA is the 4,5 unsaturated uronic acid generated by the enzyme. The galNAc residues are variably substituted with 4- or 6- or 4,6- sulfate esters.

4.1

Age-related changes in the non-enzymatic glycation of human aortic elastin

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According to one of the recent theories, age-related changes are induced by the action of free radicals and Maillard reactions (1). The aim of this study is to investigate non-enzymatic glycation of human aortic elastins, obtained from different age-groups, by measurement of AGEs, using fluorescene method, and also to determine the capacity of different aged-elastins to form fructosamine and AGEs under glycation *in vitro*. Human aortic elastins were purified from 15 age-groups of healthy accident victims, according to B. Starcher and M. Gallione (2).

Group	Age	Group	Age	Group	Age
I	under 5	VI	26-30	XI	51-55
II	6-10	VII	31-35	XII	56-60
III	11-15	VIII	36-40	XIII	61-65
IV	16-20	IV	41-45	XIV	65-70
V	21-25	X	46-50	XV	over 70

Soluble α-elastins were prepared using the method of S.M. Partridge (3). By fluorescence method we measured the levels of AGEs, expressed as arbitrary fluorescent units/mg elastin. We established that the level of AGEs increases with aging - from 7.24 AFU/mg (for the 1st group) to 22.82 AFU/mg (for the XVth group). When we measured the capacity of α-elastins from I, VII and XIV groups to form fructosamine and AGEs *in vitro* (under glycation with 100 mmol/l glucose, for 30 days), we found, that 'young' elastin has a higher capacity to form fructosamine and AGEs, compared to 'old' elastin. The levels of fructosamine were measured using the colorimetric method with NBT (4). These results suggest, that human aortic elastin, similarly to other connective tissue proteins is subject to glycation with aging. The pathogenic role of AGEs for aging process is a problem of further investigations.

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4.2

α2,3 and α2,6 linked IgG sialylation and its implications in rheumatoid arthritis

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Using *Sambucus nigra* (SNA) and *Maackia amurenis* (MAL II) lectins we have shown that serum IgG contains both α2,6 and α2,3 linked sialic acid, with the former occurring mainly on the heavy chain, and the latter on both heavy and light chains equally. The presence of α2,3 linked sialic acid was confirmed by digestion of IgG with Newcastle disease virus sialidase, which digests sialic acid in the α2,3 and α2,8 linkage, but not α2,6, followed by SNA and MAL II binding. The sialylation of IgG showed no significant differences between rheumatoids and controls, for both linkages, whilst IgG galactosylation (as quantitated with *Ricin communis* agglutinin) was significantly reduced ($P=0.001$) and *N*-acetylglucosamine (quantitated with *Bandeiraea simplicifolia II*) increased ($P<0.001$) in the rheumatoid group. IgG rheumatoid factor (RF) was quantitated by ELISA, and no correlation was found between IgG α2,6 or α2,3 sialic acid, galactose or *N*-acetylglucosamine content, in the sera or the purified IgG fractions of rheumatoid arthritis patients or healthy controls. These data suggest that the IgG oligosaccharide changes in rheumatoid arthritis are limited to galactose and *N*-acetylglucosamine. The observed changes in these sugars are a well documented phenomena that serve to validate the other aspects of this study. We are currently investigating the role that both these sialic acids, α2,3 and α2,6 may play in immune complex formation, as suggested in previous studies [1].

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4.3

Persistence of glucose (Glc) residues on oligosaccharides results in the accelerated degradation of nascent TCRα proteins within the endoplasmic reticulum: Role of Glc trimming in quantitative regulation of TCR expression during T cell development?

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Expression of the T-cell antigen receptor (TCR) complex is quantitatively regulated during T cell development with immature $CD4^+$ $CD8^+$ thymocytes expressing only 10% the number of surface αβ TCR complexes that are expressed on mature T cells. We recently found that most nascent TCRα

proteins are rapidly degraded within the endoplasmic reticulum (ER) of immature $CD4^+$ $CD8^+$ thymocytes but not mature T cells, demonstrating that stability of TCRα proteins within the ER is developmentally regulated and providing a molecular basis for low TCR expression on $CD4^+$ $CD8^+$ thymocytes. In the current study we have evaluated the role of Glc removal in regulating the stability of newly synthesized TCR proteins within the ER and their interaction with chaperone proteins in BW thymoma cells using the glucosidase inhibitor castanospermine. In addition, we examined the fate of TCR molecules in the glucosidase II deficient mutant BW cell line, PHAR2.7. Our results demonstrate that removal of Glc residues is critical for stabilizing newly synthesized TCRα proteins, but not TCR β proteins, within the ER and that Glc trimming is required for interaction of newly synthesized TCR proteins with calnexin molecules. Accelerated degradation of nascent TCRα chains induced by blockade of Glc removal severely limits $\alpha\beta$ TCR assembly in 2B4 T hybridoma cells, demonstrating that removal of Glc residues from core oligosaccharide chains is an important step in the efficient assembly of nascent $\alpha\beta$ TCR complexes within the ER. Our current working hypothesis is that quantitative regulation of TCR expression during T cell development is mediated via mechanisms involving Glc removal from oligosaccharide chains of nascent TCR proteins within the ER.

4.4

Differential clearance of glycoforms of IgG2a contribute to the increase of gal(o) in MRL mice

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To investigate the possibility that glycosylation of IgG may influence the half-life of the antibody *in vivo* and contribute to autoimmune disease, two MRL-derived monoclonal antibodies (IgG1, IgG2a), of no known specificity (no self reactivity) were utilized. Neither antibody had an oligosaccharide chain in the Fab region, as determined by papain digestion and lectin blotting. The IgG was digested with neuraminidase and B-galactosidase to remove the terminal sialic acid and galactose. The predominant IgG glycoform after digestion terminated in glucosamine [gal(o) glycoform]. Both intact IgG1 or IgG2a and the gal(o) glycoforms were iodinated and injected into the tail vein of Balb/c, NOD, MRL/+ and MRL/lpr mice. Serial bleeds during the first 50 hours were used to establish the half-life. The mean half-lives of IgG1 [both intact and gal(o)] ranged from 31 to 49 hours depending on the strain, but no significant difference was found for the two glycoforms. In contrast there was a significant difference in the half-life of the two IgG2a glycoforms, in all of the strains. Mean T½ of intact IgG2a varied from 28-38 hours (slowest for NOD), and 39-51 hours for IgG2a gal(o). There was a tendency for all of the glycoforms of IgG to be cleared faster in older MRL mice, and further studies are underway to determine whether the presence of rheumatoid factors may correlate with differences in the half-life observed with age and/or disease progression. Previous studies by Bodman et al., had shown that the gal(o) glycoform of IgG increased with age in most strains of mice. We suggest that the inefficient clearance of the gal(o) glycoform of IgG2a, possibly due to a lower affinity binding to the FcRI could contribute to this shift in glycoforms. A genetic defect in FcRI in NOD has previously been described. Since the differential clearance of the two glycoforms of IgG2a was also found for Balb/c mice, a non-autoimmune strain, it is apparent that additional gene products contribute to the autoimmune state.

4.5

Mucin of the surface coat of the parasitic nematode *(Toxocara canis)*

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Toxacara canis is a parasite of dogs, the larvae of which can infect humans causing neurological and ocular lesions as well as hepatosplenomegaly and eosinophilia in the syndrome of Visceral Larva Migrans. The infective larvae are covered in a 10-20 nm thick fuzzy surface coat. This coat is: (i) labile under conditions of immune attack, so that adhering antibodies and/or granulocytes are sloughed off, allowing the parasite to escape; (ii) carbohydrate rich and negatively charged, binding to ruthenium red and cationized ferritin; and (iii) composed of a 120 kDa serine-rich O-glycosylated antigen (TES-120). Molecular cloning of TES-120 cDNA reveals a sequence which encodes a protein of only 15.8 kDa. Its overall design is similar to mammalian membrane-associated mucins, with a signal sequence, an 86 amino acid serine-rich domain (72% serine or threonine), and a 72 amino acid C-terminal cysteine-rich domain. The TES-120 mucin contains no potential transmembrance sequence, raising the question of how the surface coat is attached to the nematode larva. One possibility is that a cuticle glycoprotein (TES-32), which is inserted in the outer membrane of the parasite and which has a 72 amino acid *N*-terminal cysteine-rich domain with strong homology to that of TES-120, acts as an anchor protein for the TES-120 mucin, in an analogous fashion to that found in some mammalian systems.

SESSION 5: Glycosylation and Inflammation I

Inflammatoy cytokines controlling acute phase protein (APP) glycosylation

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It is generally accepted that glycosylation changes of APP seen in patients' sera in disease result from changes occurring within hepatocytes and are probably due to changes of glycosyltransferase (Gn) genes expression or activity. Glycosylation changes are controlled by a net of extracellular signals comprising cytokines, their soluble receptors or steroids, independently from mechanisms regulating APP gene expression. Number of cytokines such as IL-6, LIF, TGFβ or TNF were demonstrated to be involved in mediating glycosylation alterations. Using human (Hep G2) and rat (H-35) hepatoma cell lines, and affinity electrophoresis with Con A as a ligand we demonstrated that three other cytokines - members of 'IL-6-type Cytokins' namely IL-11, *Oncostatin* M (OSM) and *Ciliary neurotropic factor* (CNTF) are also affecting profile of glycosylation of APP *in vitro*. In Hep G2 cells OSM and CNTF similarly as IL-6 and LIF decreased Con A reactivity of α1-proteinase inhibitor (PI). Effectiveness of OSM was comparable to IL-6 while CNTF had effect similar to LIF. However, addition of dexamethasone (dex) to OSM had opposite effect to that observed when dex was added to IL-6. Similarly as IL-6 and LIF in H35 cells IL-11 increased Con A reactivity of α1-acid glycoprotein (AGP). The extend of changes was comparable to that evoked by LIF. In parallel to changes in glycoslyation profile of PI in Hep G2 cells specific mRNA of α2-6 sialyl-transferase was studied. Detailed analysis of sugar structures of PI by means of serial affinity electrophoresis and HPLC with PAD demonstrated that observed changes concern mainly alterations in the branching of complex type heteroglycans.

IgG glycosylation and immune complex formation

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Changes in IgG glycosylation have been suggested to contribute to immune complex and cryoglobulin formation. Immune complexes in patients with RA involve rheumatoid factor binding to IgG. Using monoclonal rheumatoid factors derived from rheumatoid synovial cells we have found that two mechanisms contribute to the binding of RF to IgG, one of which is associated with lower IgG galactose. Seven out of 16 monoclonal RFs [Thompson *et al.*, (1988) Scand. J. Immunol 28: 501] bound better to IgG lacking the terminal galactose. This was in agreement with our findings that rheumatoid complexes expose far more detectable terminal N-acetyl glucosamine than complexes from other disease groups or non-complexed IgG, just as do those from mice with experimental rheumatological disease. Molecular modelling of the IgG Fc region has revealed the partial masking of key epitopes for RF binding by oligosaccharides in normal IgG, which are absent in RA patients. These possible mechanisms for sugar changes enhancing complex formation will be discussed.

Oxygen radicals and agalactosylation of IgG

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Several findings indicate that oligosaccharides are fundamental to the molecular recognition and clearance of human IgG. Studies over the past decade have led to the postulate that in rheumatoid arthritis there is a synthetic defect in IgG, due to a decreased B cell galactosyltransferase. In the light of evidence supporting an association between agalactosylation of IgG and the inflammatory response we have investigated: (a) the selective reactivity of oxygen radicals on IgG *in vitro*; (b) whether activated neutrophils can cause agalactosylation; (c) wether agalactosylation could be a consequence of oxygen radicals generated during an inflammatory response. **a)** Radiolytically generated peroxy (ROO•) and hyroxyl radicals (OH•), but not superoxide anion radicals (0_2^-) were found to destroy galactose on IgG. After OH• attack this was shown to be associated with an increase in the availability of *N*-acetyl-glucosamine, possibly due to its presence as a terminal residue; **b)** Exposure of IgG to human peripheral blood neutrophils caused agalactosylation, which was directly related to hydrogen peroxide production up to a concentration of 50μM. Above 50μM, agalactosylated IgG appeared to reduce probably due to the formation of aggregates, or to adherence of IgG to cells through an increased receptor interaction; **c)** In an experimental model of inflammation, induced by hydrogen peroxide, agalactosylation of IgG related temporally to degree of inflammation and to the degree of protein auto fluorescence (a specific index of oxidative damage to proteins). Our results suggest that the agalactosylation associated with chronic inflammation may not only be synthetic in nature, but may also be a consequence of post-synthetic degradation by oxygen radicals produced during inflammation.

5.1

Glycosaminoglycans in canine arthropathies

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The properties of cartilage are dependent on the collagen and proteoglycan concentration. Glycosaminoglycans (GAG), mainly chondroitin sulphate (CS) and keratan sulphate (KS) are covalently attached. Hyaluronan (HA), a high molecular weight polysaccharide, is widely distributed in connective tissues. We aim to measure GAG, KS, Chondroitin-4-sulphate (C-4-S) and HA in sera, synovial fluid (SF) from dogs with osteoarthritis (OA), rheumatoid arthritis (RA), rupture of the

cranial cruciate ligament (CCL) and normal (control). Canine antibodies to collagen I and II were measured by ELISA. Dimethylmethylene blue assay was used for the estimation of total GAG and an ELISA-inhibition assay was performed for KS, C-4-S and HA as described for use in man. Our results showed GAG levels in synovial fluids of dogs with OA and RA were significantly higher than those in synovial fluids of normal dogs ($P<0.002$). SF KS levels of RA dogs ($PP<0.001$) and serum KS levels of dogs ($P<0.03$) were significantly increased, C-4-S levels in SF of dogs either with OA or rupture of cranial cruciate ligament ($P<0.001$) and RA ($P<0.05$) were significantly higher than normal dogs. We conclude that whilst increased levels of GAG, KS C-4-S and HA were found in dogs with OA, they were not considered useful diagnostically.

5.2

Glycosylation of α_1-acid glycoprotein in hyperimmunoglobulinemia D and periodic fever syndrome: evidence for persistent inflammation

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Crossed affino immunoelectrophoresis using Concanavalin A and *Auleuria Aurantia* lectin as diantennary glycan- and fucose-specific affino components respectively, was applied to study changes in the concentration and glycosylation of the acute phase protein α_1-acid glycoprotein (AGP) in sera obtained from patients suffering from hyperimmunoglobulinemia D and periodic fever. Increases in concentration of AGP compared to control values were found not only during attacks but also during remissions. Increases were also found in the diantennary glycan content of AGP during febrile attacks, while no changes were found during remissions, compared to healthy controls. A continuous high degree of α1-3 fucosylation was accompanied by a continuous high expression of sialyl Lewis^x on AGP. Despite the clinical picture of febrile attacks with asymptomatic intervals, these studies indicate that hyperimmunoglobulinemia D should be considered a condition of persistent inflammation.

5.3

An absence of correlation between agalactosyl IgG and acute phase proteins levels in rheumatoid arthritis

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In the present study we followed the changes in acute phase response (APR) as well as in IgG galactosylation patterns in 19 rheumatoid arthritis (RA) patients. We investigated the existence of a possible correlation between some acute phase proteins (APP) and agalactosyl IgG (IgG[O]). The average values of IgG[O] at the onset of the disease and after three years of disease duration were significantly higher in RA patients as compared to healthy controls ($P<0.001$). APP serum levels were significantly higher at the onset of the disease as compared to healthy controls, but after three years of the duration of the disease all of them significantly dropped. We did not find any correlations between IgG[O] and acute phase response (APR) at the onset of the disease as well as after 3 years. For further analysis we calculated the differences between first and second measurement in IgG[O] and APR indicators. The correlation analysis between the new variables showed an association between changes in IgG[O] and ESR ($r=0.48$, $P<0.05$) as well as between changes in IgG[O] and Malya-Mace Index ($r=0.43$, $P<0.05$). No correlation between changes in IgG[O] and APPs was found.

SESSION 6: Glycosylation and Inflammation II

Activation of growth factors by heparan sulphate

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Over the past few years it has become clear that several growth factors and cytokines bind to the cell surface polysaccharide heparan sulphate (HS) and that this interaction is important for their ability to elicit a biological response in target cells. Basic fibroblast growth factor (bFGF) binds to a specific sequence (Oligo-H) in HS of structure:

GlcA - $GlcNSO_3$ - [IdoA,2S - $GlcNSO_3$]$_5$ - IdoA - GlcNAc

in which the N-sulphate groups and IdoA, 2S residues are essential for growth factor recognition. Oligo-H type sequences are now known to be the 'active sites' in HS that in some way modify the structure of bFGF enabling it to be recognised by signal transducing membrane receptors. Hepatocyte growth factor (HGF) is another growth regulatory molecule with a different structure from bFGF and which acts mainly on epithelial and endothelial cells. It also depends on HS for its biological activity. However HGF binds to a different HS sequence from Oligo-H and HS may not only modify the conformation of HGF but also act as a mechanism for directing the growth factor to its signalling receptors in the plasma membrane.

Glycosylation and maturation rate of membrane and secretory forms of human CD8α glycoprotein: implications in the activation of T-lymphocytes

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CD8 is a glycoprotein expressed on the surface of some subclasses of T-lymphocytes. It participates in the signal transduction and adhesion between T-cells and antigen presenting cells. Our previous studies on a recombinant form of human CD8α (rCD8) permanently expressed in fetal rat epithelial cells (FRT) showed that the glycoprotein has a very fast maturation rate, with a half-time of the appearance to the plasma membrane of about 30 min. During the routing rCD8 acquires 7-8 *O*-linked glycans closely spaced in the stalk region interposed between the single immunoglobulin-like domain and the transmembrane segment. Neutral and monosialylated *O*-linked chains with the branched core-2 structure are carried by mature rCD8, very likely because of the high expression of the core-2 GalNAc:β1→6GlcNAc-transferase in the host cells [1, 2].
Here we report experiments designed to elucidate the relationships between the maturation rate of rCD8 and the *O*-glycosylation process. For this purpose cDNAs encoding soluble CD8 (CD8-S) and a KDEL tagged derivative (CD8-KDEL) were expressed in the FRT cells and the kinetics of maturation and secretion were correlated with the *O*-glycosylation process. Relative to the membrane-bound form, the maturation rate of CD8-S and CD8-KDEL was much higher and only fully glycosylated forms were secreted carrying neutral and monosialylated core-2 branched oligosaccharides very similar to that of membrane-bound rCD8. The major form of intracellular CD8-S was terminally glycosylated. On the contrary the CD8-KDEL accumulated intracellularly was an intermediate form carrying mainly nascent *O*-linked GalNAc. This result indicates that the KDEL signal hinders the access of the CD8-KDEL to the medial-Golgi stack, where the core-2 GlcNAc-transferase has been located. Current results indicate that the *O*-glycosylation processing of CD8 does not correlate with the rate of routing along the exocytic pathway, rather with the host-cell glycosyltransferase pattern.
There is evidence that the activation of T lymphocytes enhances the expression of core-2 GalNAc:β1→6GlcNAc-transferase. One can thus postulate that the oligosaccharide structures that we have identified in mature forms of recombinant CD8 are those present in activated T-cells. Since the *O*-glycosylation is crucial to keep the first ectodomain of CD8 in an extended form and to avoid proteolytic degradation of the glycoprotein, the recognition function of CD8 in resting and activated T cells might be modulated by different processing of the *O*-linked chains.

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O-linked *N*-acetylglucosamine: the 'Yin-Yang' of ser/thr phosphorylation?

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O-Linked *N*-Acetylglucosamine (O-GlcNAc), a ubiquitous and highly dynamic form of intracellular protein glycosylation, was discovered in 1983 as the major terminal GlcNAc moiety in murine lymphocytes [1]. O-GlcNAc is as abundant and as responsive to cellular metabolism as phosphorylation [2]. O-GlcNAc occurs on 'hundreds' of intracellular proteins ranging from cytoskeletal-, viral-, nuclear pore-, heat shock-, and transcriptional regulatory proteins and appears to be highly immunogenic, particularly on nuclear pore proteins. The catalytic subunit of RNA polymerase II is reciprocally glycosylated or phosphorylated at its C-terminal domain [3], and virtually all RNA polymerase II transcription factors are also multiply glycosylated, suggesting an important role for O-GlcNAc in transcriptional initiation. Glycosylation-site mutants of neurofilaments suggest that O-GlcNAc mediates filament assembly [4]. Tau, a microtubule-associated protein that forms abnormal hyperphosphorylated filaments in Alzheimer's brains (PHF-Tau), is normally glycosylated by O-GlcNAc, suggesting that PHF-Tau may result from defective glycosylation at the key sites that become abnormally hyperphosphorylated in PHF-Tau.
One O-GlcNAc transferase has been purified to homogeneity from rat liver. The enzyme is a 340kDa trimer of 2-110kDa and 1-78kDa subunits, which appear to be tyrosine phosphorylated. O-GlcNAc Transferase(s) from brain appear to have

different peptide specificities compared to those from liver. A cytoplasmic, neutral *N*-acetylglucosaminidase with selectivity for O-GlcNAc peptides has also been purified from rat spleen [5]. Cloning of these enzymes is in progress. Levels of O-GlcNAc on nuclear and cytosolic proteins change within minutes of lymphocyte activation by mitogens or antigen. Overall, O-GlcNAc appears to be a regulatory modification transiently attached at sites on proteins that are similar to those used by growth regulatory, 'proline-specific', kinases. In several cases, O-GlcNAc appears to have a reciprocal relationship with protein phosphorylation.

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6.1

Reduced terminal galactosylation of the *O*-linked carbohydrates of IgA1 with raised peripheral blood mononuclear cell β1,3 galactosyl transferase activity in IgA nephropathy

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IgA nephropathy (IgAN) is a common glomerulonephritis in which IgA1 is deposited in the glomerular mesangium by an unknown mechanism. No antigen has been identified, and we have looked for glycosylation abnormalities of the IgA1 molecule as a potential non-immunological mechanism of glomerular deposition. IgA1 is one of the few serum proteins with *O*-linked glycosylation, taking the form of a hinge region series of five serine-linked *N*-acetylgalactosamine (GalNAc) moieties, further linked to terminal galactose (Gal) in the β1,3 configuration. This study investigated the *O*-linked carbohydrates in the hinge region of serum IgA1 in IgAN, using simple lectin binding assays. An ELISA-type system was designed to measure the binding of various lectins to serum IgA1 from 20 patients with IgAN and 20 matched controls. Biotinylated lectins from *Helix aspersa* (HA) and *Vicia villosa* (VV) (specific for ungalactosylated GalNAc), and from *Amaranthus caudatus* (AC) (specific for the fully galactosylated moiety) were used to assess terminal galactosylation, and the results expressed as OD at 492nm. In IgAN, IgA1 showed higher binding to the GalNAc specific lectins than control (HA 0.6 ± 0.07 vs 0.2 ± 0.05; VV 1.4 ± 0.08 vs 1.1 ± 0.06, $P<0.001$), indicating a lower degree of galactosylation of the *O*-linked moieties. There was no difference in the binding of IgAN and control IgA1 to AC lectin. By contrast, serum C1 inhibitor (C1inh), which also carries *O*-linked sugars, showed higher terminal galactosylation in 12 patients with IgAN vs 12 matched controls (VV binding 0.4 ± 0.04 vs 0.6 ± 0.03, $P=0.002$). To further investigate the underlying basis for the anomalous glycosylation in IgAN, a functional assay for β1,3 galactosyl transferase (β1,3GT) was designed. Enzyme activity was measured in peripheral blood mononuclear cell (PBMC) lysates from 12 patients and 12 matched controls, and found to be higher in cells from patients with IgAN than control (0.79 ± 0.11 vs 0.33 ± 0.006 AU/μg total protein, $P=0.001$). These results demonstrate reduced terminal galactosylation of the *O*-linked sugars of IgA1 in IgAN, which is not expressed by C1inh, another serum protein with this unusual carbohydrate type. PBMC β1,3GT activity is higher in IgAN than control, suggesting a fundamental abnormality of IgA1, which results in reduced galactosylation despite upregulation of the enzyme responsible.

6.2

IgA nephropathy: a disease of incomplete IgA1 glycosylation?

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IgA nephropathy (IgAN) is a disease characterized by the deposition of IgA1 in kidney mesangia of all patients, and by elevated serum levels of IgA1, IgA1-immune complexes (IgA-IC), and IgA1 rheumatoid factor (IgA-RF) in approximately half of the patients. Although elevated IgA, IgA-IC and IgA-RF have been described also in a number of other diseases, IgA mesangial deposits are absent or infrequent. The reoccurrence of IgAN in patients after kidney transplantation suggests that the pathogenesis is associated with changes in the molecular structure of IgA rather than with changes in the kidney mesangium. To evaluate the possible role of the carbohydrate moiety of IgA1 in this disease, we have have analyzed the carbohydrate content of IgA1 from sera of 17 IgAN patients and 11 controls. The data obtained indicated that IgA1 from IgAN patients is less galactosylated ($P=0.0085$). This galactose deficiency appears to be limited to O-glycosidically-linked glycans located in the hinge region, because IgA1 from IgAN patients exhibited decreased ability to bind *jacalin*, a lectin specific for Gal-β1,3-GalNAc glycans. Impaired binding to *jacalin* was also associated with IgA1 secreted by EBV-transformed peripheral blood mononuclear cells from IgAN patients. Furthermore, our data indicate that IgA1 from IgAN patients has increased avidity toward *Glycine max* and other GalNAc-specific lectins. When injected intravenously into mice, radioiodinated IgA1 from IgAN patients was eliminated from the circulation more slowly than that from controls. Similar results were obtained when mice were injected with radioiodinated desialylated and degalactosylated human monomeric myeloma IgA1. Examination of the distribution of degalactosylated IgA1 among organs in mice revealed decreased deposition in the liver and increased deposition in the kidney. Impaired uptake by the liver is likely caused by the decreased reactivity of degalactosylated IgA1 with the asialoglycoprotein receptor on hepatocytes, which is primarily responsible for the elimination of IgA1 from the circulation and its catabolic degradation. Increased uptake by the kidney may be explained by the presence of a GalNAc-specific receptor on mesangial cells which has been identified in the particulate fraction of mouse and human kidney cortex and on human mesangial cells.

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6.3

Changes in glycosylation of α1-acid glycoprotein (AGP) during trauma and sepsis

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To study changes in glycosylation of α1-acid glycoprotein (AGP) in sera of patients suffering from severe trauma and patients with sepsis, crossed affinoimmunoelectrophoresis (CAIE) was used. Concanavalin A (Con A) and *Aleuria aurantia* lectin (AAL) were used as diantennary glycan- and fucosespecific affinocomponents, respectively. During the first week of trauma the diantennary glycan content of AGP increased and rapidly returned to control values thereafter. The $\alpha \rightarrow 3$ fucosylation steadily increased during this first week and no return to control values was found until at least 21 days after injury. During the first week of sepsis, however, a decreasing diantennary glycan content of AGP was observed. Starting values varied among different patients and were higher or the same as control values. During the same period an increasing fucosylation was found. Changes in glycosylation of AGP are the result of cytokine-induced alterations in the hepatic synthesis of this glycoprotein. Since different combinations of cytokines are considered to be involved during trauma and sepsis, this might explain the difference in the change of diantennary glycan content during trauma and sepsis.

SESSION 7: Glycosylation and Disease I

Abnormalities in the glycosylation of IgG – its clinical utility

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It is approximately 10 years since the first detailed analysis of the variation in oligosaccharide structures attached to human serum IgG. Initial suggestions that the percentage incidence of agalactosyl structures on the bi-antennary oligosaccharide complex linked to the Fc region (Gal[0]) was increased in patients with rheumatoid arthritis have been somewhat modified. Later studies showed that raised levels of Gal[0] were largely confined to patients with RA, tuberculosis and Crohn's disease, although in other circumstances, especially in the presence of raised IL6 as occurs in the ENL reaction in leprosy patients, raised Gal[0] levels could also be identified. Studies of children with JCA and pregnant adult onset RA patients have confirmed that Gal[0] level fluctuates with disease activity and successful treatment of patients with tuberculosis is also reflected by falling Gal[0] levels. More recent studies have shown that an elevated Gal[0] in patients with early onset synovitis provides potentially useful information to the clinician in terms of predicting whether the patient will go on to develop rheumatoid arthritis and in a study of the Pima Indians this glycosylation abnormality was shown to be present prior to the onset of disease.

In our most recent study of patients with rheumatoid arthritis, followed for a minimum of four years since their first presentation to an early synovitis clinic, we have shown that a discriminate functional analysis, utilising Gal[0], grip strength age and sex, could predict the course of the disease in 94% of patients and that the Gal[0] was ranked as the most powerful parameter for this prediction.

It is clear therefore that although the debate continues as to whether Gal[0] is actually involved in the aetiopathogenesis of rheumatoid arthritis, its measurement does provide the clinician with useful prognostic information.

IgA glycosylation and disease

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In humans, IgA occurs in two isotypes, IgA1 and IgA2 with serum IgA represented predominantly by monomeric IgA1. The presence of IgA-containing immune complexes has been established in patients with primary Sjögren's syndrome (pSS), whereas IgA nephropathy (IgAN) is characterized by mesangial deposits of IgA which appears to be mostly IgA1. In search of changes in their carbohydrate moieties in both diseases, we took advantage of differential binding-properties of lectins to dissect the oligosaccharide chains attached to IgA. The proportion of sialylated IgA1 and IgA2 was augmented in pSS and, consequently, that of galactosylated was reduced. In contrast, IgA1 was the only subclass which was oversialylated in IgAN. Though originally non-significant, the difference between the amount of galactose on pathological and normal IgA became significant following treatment of the molecule with neuraminidase. Interestingly, this defective galactosylation was accounted for by the *N*-linked oligosaccharide of the IgA Fc region rather that the *O*-linked oligosaccharide of the hinge segment. Aberrant sialyl and galactosyl transferase activity warrants further investigation in diseases associated with IgA abnormalities.

IgA glycosylation in IgA nephropathies

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Human IgA glycosylation in IgA nephropathy (IgAN) was studied by the determination of its binding capacity (BC) (solid phase ELISA) to lectins: compared to normal subjects, 55% and 65% of IgA exhibited abnormal BC to PNA and to Con A, respectively, whereas 49% had abnormal PNA/Con A BC ratios. In patients with alcoholic liver cirrhosis (ALC), elevated BC to PNA was observed in 80% of patients but BC to Con A was normal. Lastly, the BC of IgA from patients with minimal change glomerular disease (MMGD) to both lectins was normal. In IgAN, an α-mannoside pre-treatment of sera strongly reduced the IgA content of their 2.5% PEG precipitates and determined a significant increase in the BC to Con A, -but not to PNA- in the patients whose circulating IgA molecules displayed low BC to both Con A and PNA, whereas the use of β-methylgalactoside induced no significant change. Further, such experiments unchanged the lectin-BC of IgA from MCGD and ALC patients. The results suggested that, in IgAN, some polymeric, partially deglycosylated, IgA molecules presenting with a great number of accessible α-mannosyl residues might form circulating non-immune complexes by binding to molecules exhibiting Con A-like activities. Such complexes might have pathogenetic significance in IgAN. Indeed, renal lesions [microscopic hematuria, glomerular infiltration by platelets and PMNs, glomerular deposition of IgA, Con A and C3 with mesangial cells (MC) exhibiting hyperactive appearance] developed one h after the injection in the rat aorta of polymeric IgA-Con A complexes, but not of polymeric IgA-PNA complexes, nor of polymeric IgA aggregates. Mesangial cells are able to recognise polymeric IgA complexes by at least two mechanisms: a binding between the MFR and accessible mannosyl residues of the Fcα domain and binding of IgA-Con A complexes through a recognition of the lectin itself. Indeed, we have observed that Con A: (a) can bind *in vitro* to MC; (b) induces a mobilization of their cytoskeleton; (c) increases their ability to endocytose gold particles and interact with zymosan or erythrocytes, opsonized or not; (d) stimulate either their lipoxygenase or their cyclooxygenase pathway according to the size of the particles tested; (e) does not induce any activation of the oxydative burst. In summary, there exists in IGAN several abnormalities of the glycosylation pattern of IgA leading to the formation of non immune complexes by binding to different (microbial? viral?, endogeneous lectin?) molecules with Con A-like properties. Such complexes are able to accumulate (a polymeric form is a prerequisite) in the mesangium but also to induce (the lectin is a prerequisite) renal lesions. This pathogenetic mechanism is relevant to the observation that evolutive episodes of IgAN are frequently triggered by bacterial and viral antigens exhibiting lectin-like activities.

7.1

Remission in pregnant rheumatoid arthritis patients is associated with decreased IgG *N*-acetylglucosamine, expression

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Seventy five per cent of patients with rheumatoid arthritis (RA) remit during pregnancy, the mechanism of which is unknown. In RA the galactose content of serum immunoglobulin G (IgG) is reduced and *N*-acetylglucosamine is increased. The aim of this study was to determine whether changes in serum IgG glycosylation during pregnancy could account for the marked clinical changes. Serum IgG was purified, using ion exchange chromatography, from 24 patients and the amount of terminal galactose (Gal) and *N*-acetylglucosamine (GlcNAc) determined by specific lectin binding. Patients all had active RA and were grouped according to those who remained unchanged ($n=6$), relapsed ($n=6$) and remitted ($n=11$). Sera were taken throughout the pregnancies and grouped into 1st trimester (1T, 1-13 weeks), 2nd trimester (2T, 14-26 weeks), 3rd trimester (3T, 27-39 weeks) and post partum (PP, within 20 weeks after delivery). IgG Gal level from the unchanged group was significantly increased in 2T, 3T and *PP* compared with 1T ($P=0.010$, $P=0.024$, $P=0.017$), while no obvious inverse relationship was found for levels of GlcNac. In contrast, IgG Gal changes were inversely proportional to GlcNac changes in the relapsed group. IgG from this group had a decreased level of GlcNac, together with an increased Gal level in the 3T compared with 2T and PP. IgG from patients who remitted during pregnancy displayed constant Gal levels throughout, while GlcNac levels decreased significantly from the 1T to 2T ($P=0.015$) and from 2T to 3T ($P=0.041$), but then increased significantly from 3T to *PP* ($P=0.005$). These three distinct patterns of glycosylation clearly relate to RA disease activity during pregnancy. The reduction in IgG GlcNac and constant Gal levels in patients who remitted could be achieved where two separate glycosylation sites are being differentially glycosylated, or a reduction in the bisecting GlcNac. The mechanism by which these glycosylation changes relate to the clinical patterns needs further investigation.

7.2

The specificity of the lectin mediated interaction of TNF-α with *Trypanosoma brucei brucei*

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It is now well established that the lymphokines and the cytokines like Il-l, Il-2 and TNF-α possess lectin-like properties [1, 2]. This new evidence of binding activity appears to be topographically distinct and at least for TNF-α has been demonstrated to be independent from the peptide portion involved in the interaction with the two previously identify 55 kD and 75 kD receptors [3, 4]. We report here that the lectin binding activity of TNF-α is able to specifically display trypanolytic activity independently from the classical tumorilytic activity of the cytokine. We have investigated the specificity of the lectinic interaction between TNF-α and *Trypanosoma brucei brucei.* (T br.br.). Chitobiose and partially processed oligosaccharide chains bearing *N*-acetylglucosamine terminated glycoproteins have the best trypanolytic inhibitory activity but no effect on the tumorilytic activity of TNF-α on the murine L929 fibrosarcoma cells used as control. We are curently characterizing the glycan structure of the endogenous receptor present at the cell surface of the trypanosome. Preliminary results using partially purified WGA fractions from T.br.br. extracts reveal that material interacting with the lectin can efficiently elicit the trypanolytic effect of TNF-α *in vitro*. The production of a specific trypanolytic agent using molecular engineering will be discussed. A detailed investigation of the lectinic properties of cytokines will open a new avenue in the understanding of the mechanism of action of these fundamental actors of the immune system.

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7.3

Ratio of neutral to sialylated oligosaccharides: a potential new tumour marker for IgG paraproteinaemias

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IgG is enzymatically glycosylated at Asn 297 in the CH2 domain of the heavy chain. We aimed to investigate whether changes in carbohydrate structure of IgG is related to malignancy in IgG paraproteins. Thirty three healthy subjects, 5 subjects with multiple myeloma (MM) and 5 with monoclonal gammopathy of unkown significance (MGUS) were followed over a 5 year period. Diagnosis of MM was made on bone marrow, clinical, radiological, and biochemical findings. All paraproteins were typed by immunofixation with specific antisera. IgG was extracted from serum using protein A affinity chromatography and purity checked by electrophoresis. Removal of intact oligosaccharides was performed by enzymatic cleavage with *N*-glycosidase with subsequent ultrafiltration. Oligosaccharide analysis was performed by high pressure anion exchange chromatography with pulsed electrochemical detection, using 100mM NaOH and Na acetate gradient from 20-250 mM. Up to 14 separate oligosaccharide peaks were identified in 3 major fractions, from each subject. The 3 major fractions were neutral, monosialylated and disialylated oligosaccharides. In control subjects neutral oligosaccharides predominated and showed a very stable pattern with time. In subjects with MGUS there was an increase in fucosylation of neutral oligosaccharides, and in patients with MM there was a significant increase in sialylated oligosaccharides. The ratio of neutral to sialylated oligosaccharides (N:S) in each group were: controls 4.26 $\pm$0.50 (SEM), MGUS 2.0$\pm$0.33 and MM 0.34$\pm$0.06. ANOVA showed a highly significant difference between all 3 groups ($P=0.001$ and $P=0.003$). We found that oligosaccharide analysis of IgG paraproteins can differentiate

clearly between control subjects, those with MGUS and those with MM. The major changes are an increase in fucosylation and sialylation of neutral oligosaccharides. The ratio of neutral to sialylated oligosaccharide may act as a useful marker of malignancy and warrants further investigation.

7.4

The glycosylation of alpha-1-acid glycoprotein in rheumatoid arthritis is correllated with the degree of activity of the disease

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Alpha-1-acid glycoprotein (AGP or orosomucoid) is a heavily glycosylated serum glycoprotein. Classified as a positive acute phase reactant, its concentration is observed to rise some two to five fold in inflammatory states and in certain other pathophysiological conditions. Moreover, the glycosylation pattern at each of its five glycosylation sites is known to alter and abnormally glycosylated variants (glycoforms) are expressed which may have functional significance in disease. In rheumatoid arthritis (RA), the heterogeneity of AGP from the sera of sufferers has been related to the degree of disease activity assessed according to the criteria of the American Rheumatism Association. Those classified as having inactive disease were observed to display no significant difference from healthy patients. However, those experiencing mild to severely active disease grades were shown to have statistically significant differences in AGP glycosylation compared to the healthy controls. Consequently, there is a progressively altered heterogeneity of AGP over the duration of RA from acute to chronic condition. A clearly defined function for AGP remains largely unknown although several studies have demonstrated immunomodulatory properties. We hypothesise that this ability to function as an immunomodulator will be determined by the glycosylation pattern and that novel AGP glycoforms associated with chronic rheumatoid arthritis are active in the altered immune regulation of the disease. Thus, we have compared the glycosylation patterns of AGP from patients with contrasting degrees of RA. This required a system capable of determining minute differences in the glycosylation patterns of AGP with sensitivity, high resolution, reproducibility and speed; all of these are achievable using high pH anion exchange chomatography with pulsed electrochemical detection.

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7.5

Agalactosyl IgG and HLA DRB1 alleles in recent onset rheumatoid arthritistis (RA) in women

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Patients with RA are more likely to have raised levels of IgG immunoglobulins lacking the terminal galactose units in the oligosaccharide chains. Elevated Gal[0] levels while not disease-specific are disease-restricted (found principally in RA, Crohn's and tuberculosis). Because there has been no prior examination of Gal[0] with HLA class II alleles, we analyzed Gal[0] and DRB1 alleles in women with recent onset RA. Patients and controls were studied from a prospective case-control study (1986-1991) of newly diagnosed RA in women aged 18-64 in the Seattle area. Gal[0] was measured for 75 patients (48 definite/classical, 27 probable RA) and 67 controls, and was considered elevated if the age corrected percentage was greater than 10%. Twenty (27%) RA cases had raised Gal[0] compared with 2(3%) of controls, $P<0.001$. HLA DRB1 alleles were determined for 59 cases. No correlation was observed between Gal[0] and heterozygosity, homozygosity, or absence of the DRB1 sequence associated with RA susceptibility. No difference was observed between probable and definite RA with the exception of a trend for association of raised Gal[0] and RF0-positivity only among definite cases (odds ratio 4.8, $P=0.07$). Clinical parameters including use of second line anti-rheumatic medications, parity, time since last live birth, and fecundability showed no relationship to Gal[0] (data not shown).

	↑Gal[0]$n=15$	Normal Gal[0]$n=44$
Sequence[a]	7/15 (47%)	26/44 (59%)
Sequence[b]	2/15 (13%)	11/44 (25%)
RF	9/15 (60%)	19/44 (43%)
XRAY[c]	1/11 (9%)	6/29 (21%)
Age of onset[d]	44 (30-62)	46 (27-64)

[a]Sequence of DRB1 shared by RA-associated subtypes of DR4, DR1 and DR14
[b]Homozygous for the DRB1 sequence
[c]Percent of those done
[d]Mean followed by range

In conclusion these preliminary data suggest that glycosylation status is not linked to DRB1 alleles in RA patients. Further investigation to assess the prognostic significance of both Gal[0] and class II alleles appears warranted in patients with new onset rheumatoid arthritis.

7.6

The value of serum analysis of agalactostyl IgG (GAL[O]) as a predictor of disease course in recent onset rheumatoid arthritis (RA)

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In a previous communication this group reported the value of GAL[O] in differentiating between patients with early synovitis who developed RA from those who did not [1]. RA has a very variable pattern of severity and in order to maximise therapeutic options in RA, the clinician requires reliable and powerful predictors of disease type at an early stage. This report describes the relative value of clinical and laboratory measures at first consultation (prior to disease modifying drugs) in one group of patients in a long term prospective

study (Group A) of recent onset RA (less than 2 years) in predicting disease outcome at 4 years. Validation was sought in a similar group from a separate but comparable prospective study (Group B). There were 14 men (25%) and 26 women (65%), total 40 patients in Group A and 8 men (33%) and 16 women (67%), total 24 in Group B. Mean age of onset was similar (50.9 and 46.5 years), as were the number with radiological erosions (83% and 67%), rheumatoid factor (RF) (90% and 78%), nodules (12% and 16%), and clinical remission at 4 years (15% and 20%). Measures of clinical activity like joint score (JS), morning stiffness (EMS) and acute phase (ESR) improved over 4 years in both groups. Mean GAL[O] levels however increased from 1.4 to 2.8 in Group A and from 2.0 to 2.6 in Group B. GAL[O] levels at 4 years were associated with ESR (r_S=0.44) and EMS (r_S=0.31), but GAL[O] at entry on its own did not have predictive value for the course of RA or eventual outcome. However, using discriminant functional analysis in Group A to identify features at entry which predicted outcome at 4 years, a combination of GAL[O] levels, age of onset, gender, functional assessments and RF titre predicted the course of RA correctly in 38 patients (94%). Thus GAL[O] has greater potential as a prognastic index early in the course of the disease than ESR.

1. Young A, *et al. Arthritis Rheum* 1991, *34 (11)*: 1425-29.

SESSION 8: Glyocsylation and Disease II

β-1,4 galactosyltransferase variation in rheumatoid arthritis

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1) Control of β-1,4 galactosyltransferase activity:
Reduced B cell β-1,4 galactosyltransferase (GTase) activity in rheumatoid arthritis (RA) is the result of a change in homeostatic control. In healthy individuals, there is an inverse relationship between B cell GTase activity and IgG galactose content. This, however, is not found in RA patients where there is a proportional relationship, which indicates a breakdown in the normal homeostatic control mechanism.
2) Are there genetic changes associated with reduced GTase activity?
RFLP analysis using 10 restriction enzymes, has revealed a Pst-I polymorphism. This polymorphism was not restricted to RA, but was also present in healthy individuals, indicating that RA associated variation in GTase activity is not as a direct result of gross structural polymorphism within the GTase gene locus. However the possibility that this and other related polymorphisms may be an integral component contributing to the multifactorial nature of RA has not been ruled out.
3) Are there intracellular inhibitors of GTase?
Using mixing experiments in both human and mouse studies, in the presence or absence of arthritis. We have demonstrated that there is no evidence to suggest the presence of a soluble intracellular inhibitor of GTase. Intriguingly, in most experiments, enhanced enzymatic activity due to synergistic action of intracellular components was detected.
4) Is there a systemic reduction in GTase activity?
The answer to this is no. Investigations using animal models of arthritis, (MRL lpr/lpr and the DBA/1 collagen induced) have demonstrated that the reduction in GTase activity is tissue specific and confined to circulating peripheral lymphocytes.
5) Are there RA specific GTase isoenzyme changes?
Cancer associated isoenzyme changes and our earlier finding that different GTase isoenzymes may account for the differential incorporation of galactose, when using a panel of different glycoprotein acceptors, suggest that changes in GTase isoenzymes may be important in RA. Observations consistent with this concept, include our recent finding that isoelectric focussing of both serum and B cell derived GTase demonstrate a possible RA associated shift in the isoenzyme profile of this enzyme.
Conclusion.
Collectively, the results presented here support our speculation that this glycosylation enzyme undergoes a variety of normal and disease associated changes, that may be both isoenzyme or tissue specific in origin. These changes may occur at the molecular level or result from post-translational modifications, and appear to contribute to the pathological processes in rheumatoid disease.

Glycosyltransferase repression: a mechanism to explain cell specific glycosylation

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The expression of specific cell surface glycan structures depends on a variety of factors, such as the activity of specific glycosyltransferases involving the presence of a functional gene, transcriptional activation, posttranscriptional control and topogenesis to the Golgi subcompartment. In addition, donor substrates (sugar-nucleotides) must be synthesized and translocated to the compartments of the secretory pathway and encounter acceptor substrates in a suitable environment.
The importance of transcriptional activation/ repression of glycosyltransferases has been highlighted by the Tn-syndrome: this rare condition is characterized clinically by mild hemolysis and immune thrombopenia and biochemically by an acquired stable repression of β1, 3galactosyltransferase (GT, E.C. 2.4.1.122) activity in a certain percentage of all hemopoietic cell lineages; affected cells express the Tn+ epitope (αGalNAc) and lack GT activity. Clones of T lymphocytes with an without GT activity were obtained *ex vivo* and shown to respond to treatment with 5′ azacytidine or Nabutyrate in reactivating GT and reexpressing the normal phenotype. These lymphocytes constitute a useful model to investigate the consequences of a defined constitutive hyposialylation.
Supported by a grant of the Swiss National Science Foundation to EGB.

Signal transduction and T cell death

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Programmed cell death (apoptosis) plays an important role in the determination of the immune receptor repertoire. How apoptosis is triggered is slowly being elucidated, and some of these pathways utilize components of mitogenic signaling networks. We have shown that at least three members of the PITSLRE protein kinase family, which are highly related to the $p34^{cdc2}$ cell cycle kinase, are involved in *Fas*-mediated T cell death. PITSLRE mRNA and protein expression are induced within 30 minutes of *Fas* antibody exposure, and further proteolytic processing of these molecules results in activated PITSLRE kinases. Treatment of *Fas*-activated T cells with the serine protease inhibitor TPCK results in the inhibition of cell death, and the accumulation of less active PITSLRE kinase precursors. Others have shown that molecular alterations of the *Fas* receptor are, at least in part, responsible for the autoimmune phenotype of the *lpr* mouse, as well as human autoimmune diseases. The mechanism underlying this autoimmune phenotype is directly related to the loss of *Fas* receptor function, resulting in the inappropriate accumulation of autoreactive T cells. Similarly, the loss of PITSLRE kinase function could lead to the interruption of programmed cell death. The normal function, and the loss of function, of the PITSLRE kinases in immune and transformed cells will be discussed, with particular emphasis on the potential role of these proteins in autoimmune disease.

Haptoglobin: a potential reporter molecule for glycosylation changes in disease

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Haptoglobin (Hp) is a secreted liver glycoprotein that binds avidly to haemoglobin (Hb) to conserve iron stores and protect the kidney from Hb damage. The glycosylation of Hp changes in many diseases (*Clin Chim Acta*, 1992, *208*, 149; *Glycosylation and Disease*, 1994, *1*, 5). The polypeptide structure of Hp is composed of two α chains and two β chains; with carbohydrate attached to the β chains. The carbohydrate component consists of four N-linked complex chains per subunit, with equal amounts of bi- and tri-antennary chains. In any normal or disease situation, there exists in the blood many forms of Hp which have minor variations in glycosylation (glycoforms). Using lectin extraction, lectin immunoassay, lectin blotting, and monosaccharide analysis, Hp glycosylation has been investigated in inflammatory diseases, liver diseases and different types of cancer. This approach can be used to detect early onset of disease, discriminate between different diseases and monitor disease progression. A very common finding is an increase in fucosylation, but the magnitude of this change varies with the disease studied, and is often accompanied by other carbohydrate abnormalities. For example, the increase in fucose is the smallest for active hepatitis and Crohn's disease and the largest for breast and ovarian cancer. However, N-acetylglucosamine content is only slightly increased or unchanged in rheumatoid arthritis or cancer, whereas in Crohn's disease and liver diseases the change is larger; and sialic acid is lower in cancer and higher in Crohn's disease and non-alcoholic liver diseases. Oligosaccharide analyses will further clarify these differences. Because Hp glycosylation provides a record of previous intracellular events, it will help to improve our understanding of the pathological processes occurring in disease and provide potential markers for clinical investigations.

8.1

Glycosylation changes and pregnancy associated remission in rheumatoid arthritis

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We have previously reported a synthetic abnormality associating reduced β-1,4 galactosyltransferase (GTase) and increased agalactosylated IgG with rheumatoid arthritis (RA). To investigate the relevance of these observations to the pathogenesis of disease, we have examined these glycosylation changes in relation to the amelioration of RA during pregnancy and the flares associated with the post-partum period, which occur in 75% and 80% of patients respectively. The enzymatic activity of GTase from the serum (S.GTase) of pregnant RA patients with active disease that were divided into three separate groups with regards to their clinical status during pregnancy (i.e. unchanged, relapse and remission), was compared. Analysis of the relationship between S.GTase and the degree of IgG galactosylation (as measured by % Lectin binding) revealed appreciable differences between the three groups. There was a significant inverse relationship ($r=-0.258$, $P<0.05$) in the remission group ($n=11$), compared with an overall proportional change in 5/6 patients ($r=+0.327$, $P<0.05$) in the relapse group and interestingly, no apparent correlation in the unchanged group ($n=6$). In contrast to these observed strict differences associated with the clinical status of the patients during pregnancy, S.GTase activity increased with advancing gestation in all three groups, and showed a marked reduction in the early post-partum period ($P<0.01$). In conclusion, these data verify our previous observation indicating RA associated disruption of a positive feedback mechanism encompassing regulation of IgG glycosylation, and perhaps of other glycoproteins, and supports the view that these glycosylation parameters are key mediators in the occurrence as well as perpetuation of symptoms in RA.

8.2

Localization of agalactosyl IgG secreting B cells in a murine model of arthritis

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It is known that agalactosyl IgG (G0) can occur as a pre-secretory event [1], but the control of this defect is poorly understood. In order to study the *in vitro* regulation of G0 production by murine B cells, we first had to identify the lymphoid compartment containing these cells. This study investigates the spontaneous, *in vitro* secretion of G0 from lymphoid compartments in MRL lpr/lpr (MRL *lpr)* and CBA/Ca mice. Cell suspensions were prepared from bone marrow, lymph node, spleen and peripheral blood, from pools of age-matched mice. Cells were cultured for 4 days and spontaneously secreted IgG measured for G0 levels using a lectin binding assay [2]. MRL *lpr* showed significantly raised amounts of G0 produced by spleen and peripheral blood cell cultures when compared with bone marrow cultures from this strain ($P<0.05$ and $P<0.01$ respectively). However, although higher, these levels were not significantly raised compared to lymph node G0. Of the 4 compartments tested, MRL *lpr* spleen and peripheral blood levels of secreted G0 were significantly raised compared to the same compartment cultures of CBA/Ca mice ($P<0.05$ and $P<0.05$). These data would suggest that in the MRL *lpr* model of arthritis, G0 is secreted mainly by peripheral blood and splenic B cells. These compartments will now be the source of cells for functional analysis of the regulation of IgG G0 by T cells and cytokines.

1. Bodman K.B., *et al Clin Exp Immunol* 1992; *88*: 420-3.
2. Bodman K.B., *et al Clin Exp Immunol* 1994; *95*: 103-7.

8.3

Mechanisms underlying the aberrant glycosylation of the MUC1 gene product observed in carcinomas

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The product of the MUC1 gene, the polymorphic epithelial mucin (PEM) is expressed on the apical surface of many simple epithelial cells. However, in carcinomas PEM is upregulated and aberrantly glycosylated which results in the exposure of normally cryptic peptide epitopes and the creation of novel carbohydrate epitopes. This may result in this molecule being seen as 'non-self' in carcinomas and so it may be a potential target for active specific immunotherapy. There is now evidence from a number of groups that a humoral and cellular immune response can be mounted to the carcinoma associated PEM. In order to study the mechanisms underlying the aberrant glycosylation of PEM a cell line (MTSV1-7) has been developed (by the SV40 immortalization of normal human milk cells) that by a number of criteria, glycosylates PEM as a normal cell. This line, in conjugation with three breast cancer cell lines (T47D, BT20, MCF-7) has been used as an *in vitro* model system to study the glycosylation of PEM. Measurement of the activity of a number of key enzymes involved in *O*-linked glycosylation showed that the activity of core 2β6 GlcNAc transferase (the enzyme responsible for the initiation of the long polylactosamine side chains observed in normal cell but not in carcinomas) is decreased by 100-fold in two of the breast cancer and by a half in the other cancer cell line. From Northern Blot analysis this reduction in enzyme activity is the result of reduced expression of the mRNA encoding core 2β6 GlcNAc transferase. These results may, in part, go to explain the short sugar side of PEM that are observed in carcinomas.

8.4

Binding of plant and animal lectins to hyposialylated human T lymphocyte clones derived from a patient with the Tn-syndrome

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The Tn-syndrome is a human disorder characterized by an acquired repression of β1, 3galactosyltransferase (GT) activity in hemopoietic cell lines. The product of GT activity is Galβ(1-3)GalNAc on O-glycans, known as the Thomsen-Friedenreich (TF) antigen. GT-deficient cells expose free GalNAc (Tn-antigen) and NeuAcα(2-6)GalNAc (sialosyl-Tn-antigen) but not NeuAcα(2-3)Gal leading to a hyposialylated phenotype on O-glycans. T cell clones with (Tn+) and without GT deficiency (TF+) have been established from a patient suffering from Tn-syndrome (Thurnher M. et al. Eur J. Immunol 1992, *22*, 1835-42). These cells were analyzed by flow cytometry using the following sialic-acid-binding lectins: Amaranthus caudatus (ACA), Maackia amurensis (MAA), Limax flavus (LF), Sambucus nigra (SNA) and wheat germ agglutinin (WGA). Controls included neuraminidase treated cells. Equal and weak binding of MAA and SNA to both TF+ and Tn+ cells was found. WGA, LF and ACA bound more strongly to TF+ cells than to Tn+ cells. Binding of ACA to TF+ cells was enhanced after neuraminidase. In summary, our data are compatible with known lectin specificities to soluble ligands. Binding of 3 murine sialic-acid-dependent adhesion molecules to Tn+ and TF+ cells was estimated using radiolabelled Fc-chimeras of sialoadhesin (Sn), myelin-associated glycoprotein (MAG) and CD22. Equal and weak binding of CD22 to both TF+ and Tn+ cells was found. Whereas binding of Sn and MAG to TF+ cells was strong (100%), binding to Tn+ amounted to 26% (Sn) and 13% (MAG). Since Sn can mediate adhesion of T lymphocytes to macrophages, reduced binding may be biologically significant. Furthermore, cytotoxic T cells are believed to be involved in demyelinating diseases. Therefore, Tn+ T lymphocytes may serve as useful controls for studies aimed at understanding the impact of SA and MAG binding to T lymphocytes.

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8.5

Studies on serum and synovial fluid IgG glycosylation in rheumatoid arthritis (RA) and its relationship to disease duration and IL6

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It is well established that IgG is abnormally glycosylated in RA, JRA, Tb and Crohn's disease. Recent studies have shown it to be abnormally glycosylated in some cases of sarcoidosis. This abnormality is measured as percentage chains lacking galactose, referred to as %G0. We have measured %G0 (using a lectin binding method) in paired serum/synovial fluid (SF) samples from 65 RA patients and 14 disease controls (including osteoarthritis, reactive arthritis, gout and back pain). RA IgG %G0 was significantly raised in both serum and SF when compared with the disease controls (P=0.02 1 and P=0.005 respectively). There was a correlation between RA serum and SF IgG %G0 levels (r=0.65) and RA SF IgG %G0 was significantly higher when compared to RA serum %G0 (P<0.001), while no significant difference was found between control serum and SF IgG %G0. RA patients divided into mild, moderate or severe disease groups showed no significant difference in %G0 between serum and SF, however, when the patients were divided into groups according to disease duration (0-2, 2-5, 5-10 and >10 years), there was a clear difference between serum and SF %G0 at 2-5 and 5-10 years (P=0.045 and P=0.035 respectively). Paired plasma and SF samples were studied in another cohort of patients (n=46) and %G0 compared with IL6 levels. Both IL6 and %G0 were raised in RA patients and while there was no correlation between serum IL6 and serum %G0, SF IgG %G0 and SF IL6 levels were highly correlated (P<0.05) suggesting that locally produced IL6 in the joint may be mediating abnormally glycosylated IgG.

SESSION 9: Carbohydrates and Therapeutics

Oligosaccharide libraries

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Two of the most difficult problems in Glycobiology are how to discover new carbohydrate ligands and how to enhance the binding affinity of such ligands once they are identified. Combinatorial chemistry has been used with great success to solve the equivalent problems in the field of peptide and nucleic acid recognition where so-called 'library'approaches have been extensively used (for a recent comprehensive review see J. Med. Chem. 37, 1233 and 1385, 1994) Implementation of a library approach for the production of biologically active oligosaccharide libraries requires decisions to be made about the identity of the monomers to be used and the size of the desired oligosaccharides that should be present in the library. The problems associated with the generation of new stereochemistry at the glycosidic linkages and of branching must also be addressed. So must the possibility of generating further diversity by functionalization, for example sulphation. The objective of our recently initiated oligosaccharide-library project is to combine the 9 most common mammalian sugars (i.e. Glc, Gal, Man, Xyl, GlcNAc, GalNAc, Fuc, GlcA and NANA) initially into all their possible trisaccharide combinations in a format useful for the discovery of carbohydrate-binding proteins like cell-surface selectins. We report here the results of our strategy of 'random glycosylation' which has been surprisingly effective in the generation random combinations of sugars.

In vivo targeting function of *N*-linked oligosaccharides

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The *N*-linked oligosaccharides on glycoproteins influence biodistribution through binding to cell specific membrane spanning lectins. In order to further establish the relationship between *N*-linked oligosaccharide structure and glycoprotein biodistribution, we have prepared *N*-linked biantennary and triantennary oligosaccharides containing a single tyrosine residue at their reducing-end and multiple terminal Gal, GlcNAc, GalNAc, NeuAc or Lewis^x determinants at their non-reducing end. The tyrosinamide-oligosaccharides were radio-iodinated and administered intravenously to mice. Serial sampling from the mouse jugular vein was used to monitor the pharmacokinetics of the oligosaccharides. Rapid clearance was observed for Gal, GalNAc, and Lewis^x terminating oligosaccharides whereas slower clearance was observed for oligosaccharides terminating in GlcNAc and NeuAc. The biodistribution of *N*-linked oligosaccharides was analyzed using whole-body-autoradiography and by direct gamma counting of dissected tissues. Liver was the major target site for Gal, GalNAc, and Lewis^x terminated *N*-linked oligosaccharides whereas oligosaccharides with terminal NeuAc or GlcNAc had no detectable target organ. The liver targeting efficiency was markedly influenced by oligosaccharide valency and terminal sugar structure. Biantennary or triantennary containing two or three terminal GalNAc residues targeted the liver with greatest efficiency whereas Gal terminated biantennary oligosaccharides only weakly targeted the liver. Lewis^x terminated oligosaccharides appeared to target a liver receptor other than the asialoglycoprotein receptor.

Studies on selectin – carbohydrate interactions

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Conjugated forms of sialyl Lewis^x-BSA have been synthesized and explored as antagonists in E-selectin/HL-60 binding assays. Increased levels of conjugation were found to lead to a three order of magnitude enhancement of inhibition compared with that of free sialyl Lewis^x (sLe^x), suggesting that cooperativity plays a role in the binding of leukocytes to activated endothelium. One of the sLe^x -BSA conjugates was explored as a reagent for monitoring surface expression of E-selectin on activated endothelial cells and found to give a staining pattern equivalent to that of an anti-E-selectin monoclonal antibody. Finally, fluorescence polarisation studies, utilizing fluorescent derivatives of sLe^x were used to measure the thermodynamic dissociation constant, K_d, for the sLe^x-E-selectin complex.

9.1

The sialic acid-dependent epitope HB-6 expressed on human lymphocytes can be reconstituted by natural and synthetic sialic acids

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Sialylation of cell surface antigens is an important regulatory element for immune responses at the level of cell-cell contacts. For example, the sialoglycoprotein CD22 binds to α2,6 sialylated structures on other cells. Recently by reaction patterns of a panel of monoclonal antibodies (mAb) it became evident that a large variety of α2,6 sialylated carbohydrate sequences are expressed on human B lymphocytes [1]. These oligosaccharides largely consist of sialylated lactosamine sequences. One of these sequences is the HB-6 epitope strongly expressed on follicular mantle zone B lymphocytes. Reaction with the mAb HB-6 was abrogated by treatment of the cells with neuraminidase from Vibrio cholerae and endo-β-galactosidase which points to a sialylated polylactosamine epitope. In order to test whether epitope recognition by mAb HB-6 is restricted to carbohydrates with terminal 5-*N*-acetyl-neuraminic acid (NeuAc) linked in α-2,6 position we desialylated surface structures on live B cells and resialy-

lated with several sialic acids employing external α2,6 sialyltransferase (a2,6ST) derived from rat liver. Resialylation of surface structures was quantitatively assessed by flow cytometric measurement of antibody binding under various conditions. Resialylation efficiency was monitored by cellular reaction with mAb 1B2 specific for nonsialylated lactosamine sequences. We found that mAb HB-6 recognized surface structures resialylated with NeuAc, NeuGc, 9-0-acetyl-NeuAc and synthetic 9-*N*-acetyl-NeuAc. Therefore mAb HB-6 recognizes α2,6 sialylated oligosaccharides without differentiation of naturally occurring sialic acids. The HB-6 epitope could be reconstituted on B cell lines Jok-1 and U266. In contrast, on cell line IM-9 which is deficient in α2,6ST and HB-6 expression the HB-6 structure could not be formed by external resialylation. This points to the requirement of cell specific precursor carbohydrate structure for the HB6 epitope. The method described here is a valuable approach to define structures of carbohydrate epitopes on live cells.

1. Schwartz-Albiez R, *et. al.*, In Schlossman *et. al., eds Leucocyte Typing V,* Oxford, Oxford University Press, 1994

9.2

Induction of endothelial sialyl Lewis^a and sialyl Lewis^x during cardiac transplant rejection

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Acute organ transplant rejection is characterized by a heavy lymphocyte infiltration. In this study we demonstrate that lymphocytes adhere to endothelium of rejecting cardiac transplants, but not to endothelium of syngeneic grafts or normal hearts analyzed with the *in vitro* Stamper-Woodruff binding assay. Concomitantly with the enhanced lymphocyte adhesion, the cardiac endothelium begins to *de novo* express sialyl Lewis^a and sialyl Lewis^x (sLea and sLex) epitopes, which have been shown to be sequences of L-selectin counterreceptors. The endothelium of allografts, but not that of syngeneic grafts or normal controls, also reacted with the L-selectin-IgG fusion protein. The lymphocyte adhesion to endothelium can be significantly decreased either by treating the lymphocytes with anti-L-selectin antibody HRL-1, or by treating the tissue sections with sialidase or anti-sLea or anti sLex mAbs. The lymphocyte adhesion can be blocked to backgroud levels when anti-L-selectin and anti-oligosaccharide mAbs are applied together. Finally, we analyzed the ability of several members of the sLex-family to block lymphocyte adhesion to cardiac endothelium. Monovalent sLex (tetramer), divalent sLex (decamer) and tetravalent sLex (22-mer) all could significantly reduce lymphocyte binding, but the tetravalent sLex-construct was clearly superior to other members of the sLex family.

9.3

Immunomodulatory activity of pichilan, a (1→3)-β-D-glucan, on macrophages

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We have studied the immunomodulatory activity of pichilan, a (1→3)-β-D-glucan, isolated from *Pichia fermentans* (CBS 187). Its structure consisted of 90% carbohydrates, including 98.5% glucose. Pichilan did not activate the complement alternate pathway. Seven and 3-day pichilan-pretreated mice were able to survive a *Klebsiella pneumoniae* experimental infection. *In vitro* experiments were performed in order to evaluate the effect of pichilan on macrophages. The reactive oxygen intermediate (ROI) release was measured by the luminal-amplified chemiluminescence test (CL), and the reactive nitrogen intermediates (RNI) by the Griess reaction. A murine macrophage cell line, MALU, isolated from C57B1/6 lpr-lpr mice lung explant was used. Pichilan alone was effective in both secretions. Consequently, MALU seems to be in a preactive state. In the same model, pichilan was able to induce IL-α and TNF-α release, measured by ELISA test. We also assessed pichilan activity on another murine macrophage cell line, J 774 A1, which was able to secrete ROI in response to pichilan. Conversely, pichilan alone could not induce RNI release; a significant nitrite liberation occurred when pichilan was combined with interferon-gamma (IFN-γ) associated or not with LPS. We did not detect any secretion in the pichilan-stimulated human monocytic cell line, Mono Mac 6. We also tested pichilan on human peripheral blood cells from healthy donors. The polymorphonuclear cells were separated from the mononuclear cells using Ficoll-Hypaque. The CL response of pichilan-activated cells was compared with opsonized zymosan-activated cell response. Both cell types release large amounts of ROI in response to pichilan. Nevertheless, the pichilan-stimulated neutrophils secreted more ROI than the stimulated mononuclear cells. The CL response varied with each blood sample tested. Therefore, our results showed that pichilan has a high immunomodulatory activity on macrophages from various origins. Pichilan can be considered as a new immunopotentiating glucan. Its advantage is a complement alternate pathway non-activation.

Aas-Eng D.A. P2.1
Adam S. P1.4
Agterberg M. P2.8
Alavi A. S8.1; P2.3, 2.5, 2.7, 4.2, 7.1, 8.1
Allen A.C. P6.1
Andrew J.M. P3.1
Arden N. P7.1, 8.1
Arican M. P5.1
Arnold C.S. S6.3
Åsheim H.C. P2.1
Atanasova M. P4.1
Austen B.M. S5.2
Axford J.S. S8.1; P1.5, 1.6, 2.3, 2.5, 2.7, 4.2, 7.1, 8.1

Baharaki D. S7.2
Bakker H. P2.8
Ball G. P7.3
Barresi F. S9.1
Bastide M. P9.3
Baumann H. S5.1
Baydanoff S.T. P4.1
Bennett D. P5.1
Berger E.G. S8.2; P8.4
Bird M. P2.2
Blomberg M.A. S6.3
Blomhoff H.K. P2.1
Bodman K.B. P2.4, 7.6, 8.2, 8.5
Bonatti S. S6.2
Bond A. S5.2; P1.5, 4.2, 7.1, 8.1
Bradfield P. P3.2, 3.3
Branchaud A. P7.5
Brenchev I. P4.1
Britten C. P2.2
Brockhausen I. P8.3
Brózik M. P1.1
Broschat K. S9.3
Brown S.T. P8.1
Burchell J. P8.3

Carrington S.D. P1.4
Carter S.D. P5.1
Childs R.A. S3.3
Chiu M. S9.2
Chou T-Y. S6.3
Clarke J.L. S2.3
Cole R.C. S6.3
Comer F.I. S6.3
Constable P. P1.2
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