Second Jenner International Glycoimmunology Meeting

An international meeting of scientists from Europe, the USA, and Japan to discuss the role carbohydrates play in shaping the immune response and how this may be associated with disease mechanisms, held on 1 and 2 November 1992 at St George's Hospital Medical School, University of London, United Kingdom.

(Organising committee: John S Axford (chairman), Frank C Hay, Azita Alavi, Angela Bond, Martin Dalziel, Meinir Jones, Michael Lind, Andy Soltys, Sue Henderson (administrator))

Methodology and glycoproteins

Mass spectrometric strategies for characterising glycoproteins

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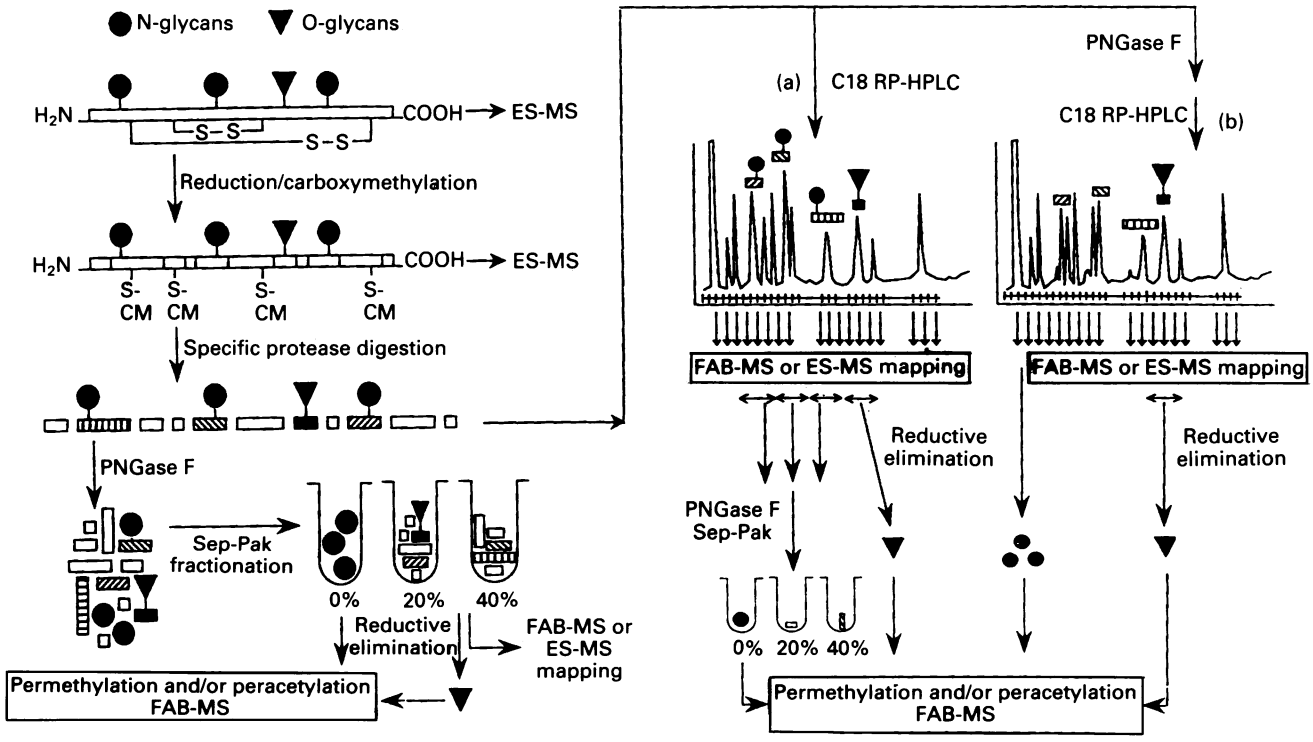
High field magnetic sector double focusing mass spectrometry has had a major role in biopolymer analysis in the 15 years since it was pioneered by one of us (HRM), and high field instruments fitted with fast atom bombardment (FAB) sources have solved a wide range of glycoprotein structural problems. More recently, a powerful new method for analysing intact macromolecules, electrospray mass spectrometry (ES–MS), has been successfully applied to glycopeptide and glycoprotein analysis. In this paper we review the types of problems amenable to FAB–MS and ES–MS and highlight the most effective strategies for studying glycoproteins.

In the FAB experiment quasimolecular and fragment ions are sputtered, by a beam of accelerated atoms or ions, from a viscous matrix containing the sample under examination. The quasimolecular ions contain compositional information while the fragment ions are structurally informative. As a general rule glycans whose hydroxyl groups are protected by functional groups, such as methyl or acetyl, fragment more reliably than their native counterparts. In addition, derivatised samples can be readily freed from impurities such as salt that may compromise the FAB experiment and often afford better quality data than their native counterparts. Consequently, derivatisation underpins most successful FAB–MS strategies in the glycoprotein area. Permethyl derivatives, in particular, are widely used in the characterisation of N- and O-glycans because of the highly specific fragmentation that occurs at aminosugar glycosidic linkages, affording characteristic 'maps' of fragment ions that define the compositions of all non-reducing epitopes.

ES–MS is a method by which a stream of liquid containing the sample of interest is directly injected into the atmospheric pressure ion source of a mass spectrometer. A spray of microdroplets is generated which is then stripped of solvent to give multiply charged species that are most commonly analysed with a quadrupole detector. The mass spectra show a distribution of signals carrying varying numbers of net positive or net negative charges reflecting the number of ionisable groups in the biopolymer. In the glycoprotein field ES–MS is most useful for defining the molecular weights of intact glycoproteins or glycopeptides.

FAB–MS, together with ES–MS in some instances, is a powerful tool for defining the following structural features in glycoproteins: (*a*) the degree of heterogeneity and type of glycosylation, eg high mannose, hybrid, complex, etc; (*b*) sites of glycosylation; (*c*) branching patterns; (*d*) the number and lengths of antennae and the patterns of substitution with fucose, sialic acids, or other capping groups such as sulphate; (*e*) the sequences of all non-reducing structures irrespective of their overall size or the amount of heterogeneity; (*f*) the presence of *N*-acetyllactosaminoglycan chains and determination of the maximum number of repeats; (*g*) the fidelity of the carbohydrate structures of recombinant glycoproteins; (*h*) detection of abnormalities resulting from defects in biosynthesis; (*i*) the composition of both the glycan and the lipid moieties in GPI anchors and the sequences of the glycans; (*j*) the degree of modification of nuclear and cytoplasmic proteins with O-GlcNAc and the locations of O-GlcNAc attachment.

The figure illustrates a general strategy that we have developed for investigating N- and O-glycan structures, which exploits the strengths of both FAB–MS and ES–MS. The protocol, shown on the left side of the figure, yields data



Strategies for analysing glycoproteins by fast atom bombardment mass spectrometry (FAB–MS) and electrospray mass spectrometry (ES–MS). [Reproduced by permission of Oxford University Press from Glycobiology: Practical Approach (Fukuda M, ed) Oxford, Washington DC: IRL Press.]

on the total glycan population, whilst the strategy, on the right, which incorporates high performance liquid chromatography, is pursued when site-specific glycosylation is being investigated.

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Biochemical analyses of carbohydrate-protein interactions

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Recognition of carbohydrates is an important event in biological systems. Therefore, carbohydrate-protein interactions merit detailed studies. The interactions can be measured by gel filtration, ammonium sulphate precipitation, membrane analysis, or microfuge centrifugation through density media. More sophisticated instrumental analyses include capillary zone electrophoresis and plasmon sensor (BIAcore). The binding data are best analysed directly by non-linear regression rather than after transformation for linearisation.

Thorough analysis of carbohydrate-protein interactions requires studies on the structures of carbohydrates (ligands) and of proteins (receptor). Protein structures can be studied by a range of available techniques, such as sequencing, nuclear magnetic resonance, and when possible, *x* ray crystallography. Molecular biology plays an important part in gaining insight into the protein structure by producing many mutants.

For the carbohydrate ligands the highest degree of structural purity is necessary for sound, in-depth studies of interactions. This can be achieved by thorough purification of naturally derived glycoconjugates by highly effective methods of separation, such as various forms of high performance liquid chromatography (table).[1]

Alternatively, structurally unambiguous glycoconjugates can be synthesised by well defined procedures. These include conjugates of carbohydrate derivatives to proteins, lipids, and non-biological carriers and polymers.[2] These conjugates were shown to be extremely useful in the studies of carbohydrate binding proteins from animals. Hepatic carbohydrate receptors belong to the C-type binding proteins because they require calcium for binding activity. They recognise only the terminal sugar residues of the oligosaccharide chains. Although hepatic carbohydrate receptors from different species show different sugar specificities, there are some common features in the requirement of the substituents of the sugar rings. For example, 3-OH and 4-OH are the most important, and 6-OH is unimportant.[3]

One most outstanding feature manifested by a number of animal carbohydrate binding proteins is their sensitivity to branched oligosaccharide structures and the drastic enhancement of binding affinity by clustering of target glycosides. This is understandable because branching is one of the structural features that distinguishes carbohydrates from other biopolymers. For optimal binding, clustering of the target sugars must be in proper geometry.[4] By knowing the optimal geometry of the target sugars in cluster, it is possible to prepare relatively small carbohydrate ligands of relatively strong binding affinity.[5]

One of the powerful methods for determining conformational structure that can be related to the ligand activity is the fluorescence energy transfer technique. We attached a naphthyl group at the N-terminus of a triantennary glycopeptide and modified one of the terminal galactose residues with a dansyl group to perform the energy transfer. Measurement of fluorescence lifetime showed that one of the three branches is quite inflexible and the other two more flexible.[6] These data explain many of our previous binding data.

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FACE: fluorophore assisted carbohydrate electrophoresis. A new method for the anaysis of complex carbohydrates

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Numerous important biological processes are mediated by the glycans of glycocojugates. Their structural analysis presents considerable difficulties both because of the small quantities which are available from biological sources and because of the inherent difficulty of analysing their complex structures. To study these problems a new method of analysis, FACE (fluorophore assisted carbohydrate electrophoresis), has been developed in which reducing saccharides are labelled quantitatively with a fluorophore and the derivatives separated with high resolution by polyacrylamide gel electrophoresis (PAGE). The resulting electrofluorograms can be viewed with high sensitivity using an imaging system based on a cooled charge coupled device. Subpicomolar quantities of individual saccharides can be detected. Two fluorophores, 8-aminonaphthalene-1,3,6-trisulphonic acid and 2-aminoacridone, have been used to label a variety of oligo- and monosaccharides.[1-3] Both fluorophores enable the electrophoretic separation of a variety of positional isomers, anomers, and epimers. After labelling with 2-aminoacridone, glucose and all of the six monosaccharides found commonly in mammalian glycoproteins could be separated in a single gel lane. 2-Aminoacridone can also be used to distinguish unequivocally between acidic and neutral oligosaccharides. Mixtures of oligosaccharides obtained by enzymatic cleavage from a variety of glycoproteins have been labelled with the alternative fluorophore, 8-aminonaphthalene-1,3,6-trisulphonic acid, and separated by high resolution PAGE to yield an oligosaccharide profile of each protein. The method enabled rapid, simultaneous, parallel analyses of multiple samples. Preparative electrophoresis was used for the isolation of the individual labelled oligosaccharides for further analysis. Methods for obtaining saccharide sequence information in the isolated oligosaccharides have been developed using enzymatic degradation and FACE.

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Binding of synthetic and natural branched Gal/GalNAc cluster ligands

Valency	*Cluster ligands*	I_{50} (μmol/l)*	
		Solubilised CBP‡	*Intact hepatocytes*
1	LacAH†	260	40
	GalNAcAH	5	4
2	$Asp(LacAH)_2$	7	0·6
	Asialo biantennary glycopeptide		2·4
	$Asp(Gly–GalNAcAH)_2$	0·08	0·04
3	$TyrGluγGlu(LacAH)_3$	3	0·05
	$TyrGluγGlu(GalNAcAH)_3$	0·004	0·0002
	Asialo triantennary glycopeptide		0·004

*I_{50} = 50% inhibition point.
†AH = 6-aminohexyl.
‡CBP = carbohydrate binding proteins.

Epitope modelling in glycoimmunology

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During the 1980s, series of hybridoma derived monoclonal antibodies became available which had been raised against cells in various stages of differentiation and neoplasia. Many of these were subsequently shown or suspected to recognise carbohydrate structures as either components of glycoproteins[1] or lipids[1 2] covering the cell surface. Characterisation of the specificity of these monoclonal antibodies largely relied on the use of oligosaccharides of known structure, purified from milk and other sources, as inhibitors of the binding of the monoclonal antibodies to cells or glycoprotein fractions.[1 3] In this way a series of

oligosaccharide oncodevelopmental antigens was defined at the structural level. These included differentiation antigens of cells of the haemopoietic stem cell lineage, the oligosaccharide structure of which or related oligosaccharide sequences are now being ascribed functions in lymphocyte migration.[4]

PHYSICOCHEMICAL ANALYSIS OF OLIGOSACCHARIDES

Structural elucidation of oligosaccharides recognised by antibodies or carbohydrate binding proteins (lectins) has been carried out by high performance liquid chromatographic techniques,[3 5 6] mass spectrometry,[3 7] and nuclear magnetic resonance (NMR) spectroscopy.[3 8] Information from NMR spectroscopy can be interpreted to give conformational information[8 9] as follows. As has been shown by our NMR studies on oligosaccharides with H, A, B, Le^a, Le^b, and Le^x (SSEA-1) antigens,[8-10] comparison of the chemical shifts of series of structurally related oligosaccharides can be used to predict the chemical environment of functional groups and the proximity of proton–oxygen atoms. This information provides a set of possible confirmations with alternative orientations of the monosaccharide constituents. Complementary information on through space proton–proton distances from NMR nuclear Overhauser enhancement studies can then be used to distinguish between possible solution confirmations by incorporating the date as distance constraints in molecular modelling by computer graphics. The NMR experiments give one, two, and three dimensional proton detected spectra (combinations of COSY, HMQC, TOCSY, ROESY), which provide comprehensive assignments of chemical shifts and conformational data in aqueous solutions.

MOLECULAR MODELLING OF OLIGOSACCHARIDES BY COMPUTER GRAPHICS

Computer graphics are carried out using a personal IRIS workstation and Biosym software (or equivalent). Parameters for oligosaccharide structures are introduced into the commercial programs[8] compatible with glycoprotein modelling and amino groups. A monosaccharide library is set up using coordinates from the Cambridge crystallographic database.

Disaccharides are explored for low energy minima and oligosaccharides built up at ϕ, ψ angles in lowest energy minimum wells. The solution conformation of small oligosaccharide determinants has been studied by molecular dynamics in water boxes under periodic boundary conditions; larger molecules are manipulated in vacuo with inclusion of variable dielectric constants to simulate a water environment.

MOLECULAR MODELLING OF GLYCOPROTEINS BY COMPUTER GRAPHICS

Protein modelling is carried out by incorporating sequences of high probability of forming α helices, β strands, and β turns as predicted by standard algorithms and by homology.[11] Peptides with predicted secondary structure or biological/immunological activity, or both, are analysed by NMR as described for oligosaccharides. Information is also included from NMR analysis of glycopeptides. Where no secondary structure can be predicted, protein sequences are allowed to fold using molecular dynamics simulations. Oligosaccharides are added at predicted glycosylation sites[12] and the conformational space occupied around the protein is explored by the graphics.

In this way features of the protein which can be seen by antibodies for B cell recognition are predicted. Similarly, areas of the molecule capable of being processed for major histocompatibility complex restricted peptide presentation are established. These studies are also being used to determine the role of oligosaccharide components in T cell recognition including the possibility of these components affecting superantigen activity in tuberculosis.[13]

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The immunoglobulin supergene family

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Glycosylation changes have been observed on the IgG molecules in patients with rheumatoid arthritis (RA). IgG glycosylation changes in RA are manifested by disclosing the presence of *N*-acetylglucosamine on the Fc oligosaccharides instead of the normally terminating galactose or sialic acid. The variable portion of the IgG molecule may also be glycosylated and there is some evidence suggesting that in patients with RA the $F(ab')_2$ portion has increased levels of sialic acid. This increase in the $F(ab')_2$ levels of sialic acid was found to be crucial for complex formation. It has been suggested that the sialic acid in the variable part of the molecule fits into the pocket made available by the lack of terminal galactose on the IgG Fc region of IgG.

We used two approaches to analyse oligosaccharides on soluble and complexed IgG. The first uses sodium dodecylsulphate polyacrylamide gel electrophoresis of soluble and complexed IgG and subsequent analysis of the heavy and light chains using a panel of lectins. The second approach establishes the antigenicity of a panel of IgG molecules with varying degrees of glycosylation. We determined the binding of monoclonal IgM rheumatoid factors derived from human synovial tissue to a panel of IgG molecules. We conclude that the glycosylation of IgG can have an effect on the antigenicity of the IgG molecule.

Our work has concentrated predominantly on the defective glycosylation on the IgG molecule. Glycosylation changes have also been found, however, on the acute phase proteins present in patients with RA. Other glycoproteins may also show a change in their glycosylation in patients with RA, and we are currently investigating whether these molecules may be responsible for the trafficking of lymphocytes into inflamed joints.

Inflammation induced expression of sialyl Lewis X groups on human α_1 acid glycoprotein

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Inflammation induces characteristic changes in the glycosylation of α_1 acid glycoprotein (AGP) and other acute phase glycoproteins with respect to the degree of branching of the N-linked glycans, as revealed by their reactivity with concanavalin A. Cytokines, such as interleukin 1, tumour necrosis factor, and interleukin 6, involved in the induction of the inflammatory reaction, have been shown to be responsible for these changes by affecting the glycosylation process in the liver.[1 2]

We now report inflammation induced changes in the fucosylation of AGP of the type Lewis X ($Gal\beta1\rightarrow4(Fuc\alpha1\rightarrow3)GlcNAc$–R), as detected by the fucose binding *Aleuria aurantia* lectin (AAL) in crossed affinoimmunoelectrophoresis of human serum samples. The absolute amount of Lewis X substituted AGP molecules and the number of Lewis X determinants per molecule were enhanced under various acute inflammatory conditions (fig 1) and in patients with rheumatoid arthritis. The number of AGP molecules expressing three or more Lewis X substituted glycans (represented by the most strongly retarded fractions) was especially increased. A considerable part of the Lewis X groups appeared also to be substituted with an $\alpha2\rightarrow3$ linked sialic acid residue, at least during acute inflammation. This was established by staining with the monoclonal antibody CSLEX-1, directed against sialyl Lewis X determinants (SLEX), of western blots containing equal amounts of AGP isolated from the serum samples of a patient before and at various days after laparotomy (fig 2). Maximum staining with CSLEX-1 was observed on the second day after the operation, remaining at a high level for subsequent days. The abundant staining at

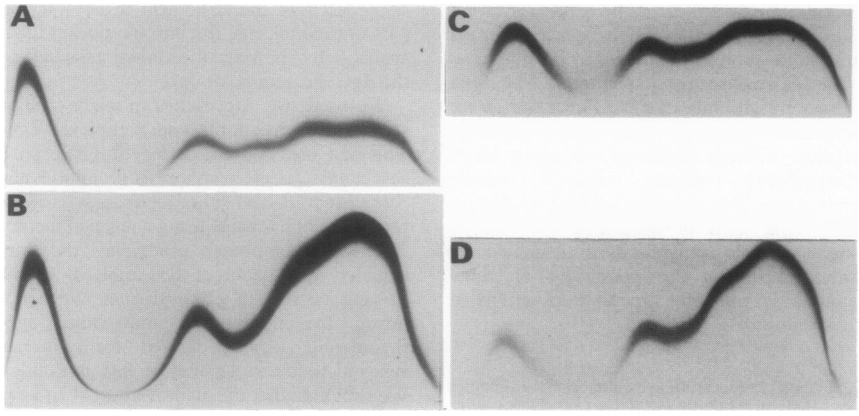

Figure 1 Reactivity of human α_1 acid glycoprotein (AGP) with Aleuria aurantia lectin (AAL) in serum of a patient one day before (A) and four days after laparotomy (B), and of a burn patient one day (C) and 30 days (D) after injury. A 1 µl aliquot of serum was analysed by crossed affinoimmunoelectrophoresis with AAL as the affinocomponent in the first dimension gel and rabbit antihuman AGP IgG in the second dimension gel. Only the second dimension gels are shown; the application site in the first dimension gel coincides with the right hand side of each figure.

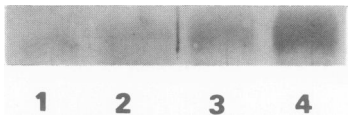

Figure 2 Inflammation induced increase in the expression of SLEX antigens on α_1 acid glycoprotein (AGP). A western blot of immunoaffinity purified preparations of AGP from the serum of the laparotomy patient of fig 1 was treated with CSLEX-1, a mouse IgM monoclonal antibody. Bound molecules were detected using goat antimouse IgM alkaline phosphatase conjugate. Care was taken that the same amount of AGP (8 µg) was applied in each lane. Only the part of the blots containing the AGP bands is reproduced. Lane 1, AGP isolated from pooled human serum; lanes 2, 3, and 4, respectively one day before, and one and two days after laparotomy.

these time points is in sharp contrast with the weak staining of AGP before the operation, and of AGP isolated from pooled human serum. It can be concluded, therefore, that inflammatory conditions can induce a strong increase in SLEX substituted AGP molecules, which persists at a high level throughout the inflammatory period.

The SLEX structure is of special interest because, when present on leucocytes, it is the ligand for the cell adhesion molecules E-selectin (the endothelial-leucocyte adhesion molecule ELAM-1) and P-selectin (GMP140/PADGEM/CD62), which affect the inflammation dependent adhesion of neutrophils, monocytes, or resting T cells to vascular endothelium or platelets.[3] E-Selectin and P-selectin are normally not expressed on the surface of the cells, but can be induced rapidly on stimulation with inflammatory mediators. Walz *et al* [4] have reported that AGP has an affinity for E-selectin and that this affinity can be substantially enhanced by in vitro fucosylation of AGP, most probably increasing the expression of SLEX groups on the glycans. Therefore, our results suggest that the inflammation induced increase in SLEX substituted AGP molecules represents a physiological feedback response on the selectin mediated interaction between leucocytes and inflamed endothelium.

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Glycosylation profiles of chimeric human/mouse antibodies produced in vivo and in vitro

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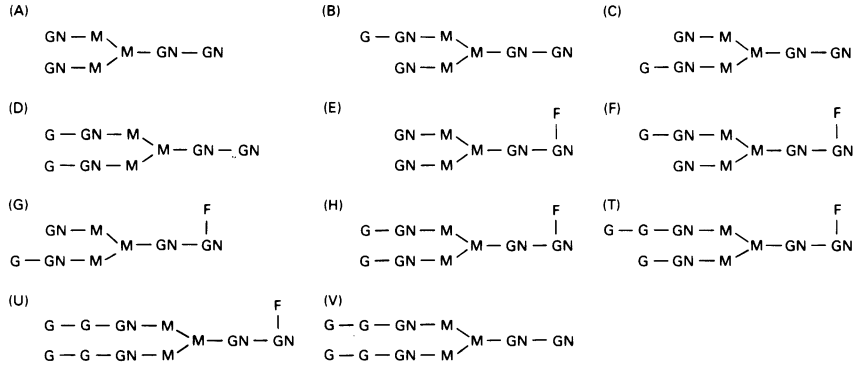
The human IgG molecule has a single conserved glycosylation site within the C_H2 domain. The oligosaccharide moiety is of the complex type and constitutes 20% of the mass of the domain. It overlays the Fx face and appears to be accommodated within the internal space between the two C_H2 domains. Multiple non-covalent interactions between the sugar residues and amino acid side chains suggest that the carbohydrate contributes to the tertiary structure of the C_H2 domain, and hence the Fc region, and consequently functional activity, at least indirectly.[1]

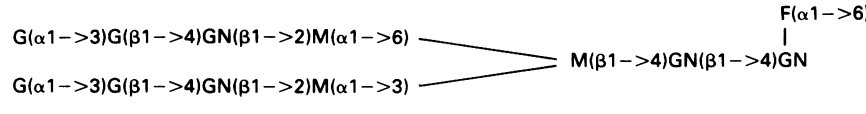
For IgG molecules glycosylation at the acceptor site (asparagine 297) always occurs, though the extent of completion of the outer arms is extremely variable, giving rise to differing glycoforms. Considerable heterogeneity of glycosylation is observed for IgG molecules that are the products of a single IgG secreting clone.[2] As it has been shown that aglycosylated IgG lacks the recognition structures necessary for interaction and activation of all three human Fc receptors[3] and the C1 complement component[4] it is important to determine whether differing glycoforms express differing biological activities.

Oligosaccharides derived from chimeric anti-NIP IgGs of the four subclasses produced in hollow fibre bioreactors, and IgG3b produced by three separate growth conditions. Neutral oligosaccharide fractions from chimeric IgGs of the four subclasses were aminated and subjected to high performance liquid chromatography on an ODS silica column. The percentage of each component is given in the table

	IgG1 hollow fibre	*IgG2 hollow fibre*	*IgG3b hollow fibre*	*IgG3b ascites*	*IgG3b stills*	*IgG4 hollow fibre*
A	4·0	6·4	5·0	1·2	—	2·6
B	4·3	2·4	3·5	3·1	2·5	4·2
C	2·4	3·9	2·8	3·0	1·5	4·3
D	2·3	3·7	3·3	4·7	9·1	2·3
E	18·9	35·4	25·3	19·9	2·3	20·8
F	25·3	14·6	25·0	29·1	15·0	20·1
G	9·5	19·2	12·7	13·7	5·2	19·6
H	26·9	12·8	15·4	23·1	39·2	21·6
T	2·3	1·2	3·5	2·2	7·9	1·5
U	3·0	0·4	2·5	—	13·5	3·0
V	1·0	—	0·8	—	3·8	—



Structures of oligosaccharides derived from anti-NIP IgGs.
Key: G=galactose; GN=N-acetylglucosamine; M=mannose; F=fucose. Peak (U), for example, represents:



Oligosaccharide analysis of chimeric human/mouse antibodies produced in the mouse J558L cell line reveals an essentially mouse profile and an absence of molecules bearing a bisecting *N*-acetylglucosamine (table, fig, p 1272). A further mouse characteristic is the presence of galactose (α1–3) galactose residues. This structure is not present in human glycoproteins and it is reported that human serum samples contain antibodies to this structure. Clearly the mouse cell is determining the glycosylation pattern.

As the human IgG2 has a different profile of galactosylation (F<G), also observed in human IgG2 myeloma proteins,[2] and as the general preference F>G is opposite to the galactosytransferase preference, it is clear that the heavy chain template can also dictate the glycosylation pattern. We have also shown that the proportions of the differing glycoforms produced are sensitive to the culture conditions used, such that IgG3 produced in still culture results in higher levels of galactosylation than when produced in hollow fibre bioreactors or in vivo as an ascites tumour. It is apparent, therefore, that parameters influencing glycosylation include the tertiary/quaternary structure of a protein, the complement of glycosyltransferase present within the cell in which it is synthesised, and the conditions under which the cells are cultured.

Modelling of the Fc region of the IgG molecule allows rationalisation of some of the functional variations observed for differing glycoforms. Thus amino acid residues contributing to Fc receptor and C1 recognition have been localised to the C_H2 domain and they have been shown to be sensitive to glycosylation. In contrast, recognition sites for rheumatoid factor and staphylococcal protein A binding include residues of both the C_H2 and C_H3 domain that are in a different plane from that occupied by the carbohydrate, and interactions with these ligands are not obviously different between differing glycoforms. We have observed significant differences of interactions between rheumatoid factor and aglycosylated IgG, however.

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Cell surface carbohydrates in the immune response

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Glycosylation of the lymphocyte surface changes during cell activation. Study of the saccharides of cell surface glycoproteins has been neglected, but such residues may have a major functional role. We therefore investigated the expression of carbohydrate determinants on the surface of rat lymphocytes by flow cytometry using a panel of fluorochrome labelled lectins. Certain sugars were found to be distributed differentially on lymphocyte subpopulations and on activated and normal cells. At least two new carbohydrate structures appear on activated T cells, and their partial structures were inferred from the lectin binding data.

Seven panels of lectins were used to phenotype normal lymph node cells from AO ($RT1^u$) rats as follows:

Panel 1 (mannose binding)
- GNA —*Galanthus nivalis*
- Con A —*Canavalia ensiformis*
- PSA —*Pisum sativum*
- LCA —*Lens culinaris*
- VFA —*Vicia faba*
- PHA e and l —*Phaseolus vulgaris*, isolectins e and l

Panel 2 (*N*-acetylglucosamine binding)
- DSA —*Datura stramonium*
- LEA —*Lycopersicon esculentum*
- PWM —*Phytolacca americana*
- STA —*Solanum tuberosum*
- WGA —*Triticum vulgaris*

Panel 3 (β-galactosamine binding)
- ECA —*Erythrina cristagalli*
- PNA —*Arachis hypogaea*

Panel 4 (*N*-acetylgalactosamine binding)
- BSI-A4 —*Griffonia simplicifolia*, isolectin A4
- BPA —*Bauhinia purpurea*
- CAA —*Caragana arborescens*
- CFA —*Codium fragile*
- DBA —*Dolichos biflorus*
- HAA —*Helix aspersa*
- HPA —*Helix pomatia*
- SBA —*Glycine max*
- PLA —*Phaseolus limensis*
- SJA —*Sophora japonica*
- VVA-A2B2 —*Vicia villosa*, isolectins A2 and B2
- WFA —*Wisteria floribunda*

Panel 5 (α-galactose binding)
- BSI-B4 —*Griffonia simplicifolia*
- MPA —*Maclura pomifera*
- MCA —*Momordica charantia*

Panel 6 (fucose binding)
- LTA —*Tetragonolobus purpureus*
- UEA-1 —*Ulex europaeus*

Panel 7 (sialic acid binding)
- MAA —*Maakia amurensis*
- SNA —*Sambucus nigra*

The staining with the fucose binding lectins was negative. Most of the other lectins divided the cells into at least two populations, one either negative or less positive than the other.

The expression of carbohydrates was studied on B and T lymphocytes before and after they had been activated in an allogeneic mixed lymphocyte reaction. Monoclonal antibodies against CD5 (OX19), MHC class II (OX6), CD4 (W3/25), CD8 (OX8), and the interleukin 2 (IL-2) receptor (OX39) were used to identify cell populations in dual colour fluoresence analyses. The GalNAc lectins (panel 4) were studied in detail. Before activation all the B cells stained strongly with the GalNAc lectins while the T cells did not. After activation the T cells expressed saccharides which were detected by the GalNAc lectins. Competitive lectin binding studies and a knowledge of the fine specificity of the lectins were used to derive a possible structure for the glycan appearing on activated T cells. The best possible fit with the data is of a bifurcating glycan (*glycan I*) with two identical branches consisting of (GalNAc α1, 3Gal β1,3GlcNAc β1-)$_2$=R–.[1] Sips analysis of the flow cytometry data on the titration of activated T cells with BPA showed that it bound with moderate affinity to glycan I and with higher affinity to another glycan, possibly Gal β1,3GalNAc α1–, expressed on all lymphocytes.

Competition analysis further showed that a second glycan (*glycan II*), which bound ECA and BSI-B4, appeared in parallel with glycan I on activated T cells, but was distinct from it. The putative sequence is Galα1, 3Gal β1, 4GlcNAc β1– and it lies within 85 Å of glycan I, probably in fixed orientation to it. A third set of glycans (*glycan III*) also appeared on activated cells, was distinct from glycans I and II, was more than 90 Å from them, and bound DSA, LEA, PWM, STA, and WGA. It probably contains multiple *N*-acetyllactosamine residues.

A study of the kinetics of the appearance of glycan I during activation showed maximal expression after four days in the mixed lymphocyte reaction, precisely coincident with the expression of the high affinity IL-2 receptor. The IL-2 receptor, however, is a marker for cell activation and thus this result does not imply that the lectins are directly binding to the IL-2 receptor itself. The CD45 molecule was of interest as it has many splice variants which may or may not include certain glycosylation sites. A study of the expression of the heavy molecular weight isoform, CD45RC (defined by the monoclonal antibody OX22 in the rat) showed that the CD45RC molecule appeared before the glycan and was lost before it too. Hence the temporal dissociation of the expression suggests that the GalNAc lectins are not binding to the CD45 glycoprotein. Further studies will be required to determine the proteins to which these glycans are bound, and any function they might have. However, the control of expression of certain specific glycan structures on activated T cells strongly suggests that these saccharides do have a functional significance.

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The glycosyl transferases

CD antigens and α-2,6-sialyltransferase

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The lymphocyte antigen CD75 is expressed on the surface of COS cells after transfection of the α-2,6-sialyltransferase gene.[1] Several monoclonal antibodies (mAbs) directed to CD75 recognise antigenic determinants which are destroyed after treating lymphocytes and red cells with neuraminidase from *C perfringens*.[2] These findings show that the structures recognised by CD75 mAbs comprise sialic acid and are produced by the enzyme α-2,6-sialyltransferase. The cell surface molecules which carry the carbohydrate determinants recognised by CD75 mAbs are not primary products of the α-2,6-sialyltransferase gene,[1] and are uncharacterised. Antibody binding studies,[2] however, suggest that CD75, like some other cell surface molecules including Thy-1, is expressed in a number of immunochemically discrete subsets or 'composite glycoforms'.[3] At least one CD75 antigenic

determinant is concealed by sialic acid and is only accessible to antibody binding after neuraminidase treatment of lymphoid cells.[2]

Probably as a reflection of tissue-selective expression of specific glycoforms, different CD75 mAbs give different staining patterns on lymphocytes[2] and non-lymphoid tissues, including liver hepatocytes, bile canaliculi, and intestinal epithelium (Andrew J M, Guy K, unpublished results). On all mature B lymphocytes CD75 is expressed at high levels and on T cells CD75 is a new subset marker. About 40% of CD4 positive T lymphocytes from normal peripheral blood express CD75, and the antigen is found predominantly on cells devoid or low in expression of CD29 (VLA-β1 integrin), CD54 (ICAM-1), CD58 (LFA-3), and CD45RO (leucocyte common). CD75 is expressed on lymphocytes high in expression of CD45RA. These data show that CD75 is a marker of naive or quiescent T lymphocytes. Not all CD75 mAbs react with significant numbers of peripheral blood T cells, however, and the expression of the antigen is altered dramatically by mitogen activation.[2]

CD75 is expressed on red blood cells from all normal adult donors and, on red cells, the antigen is upregulated by the product of a dominant inhibitor gene called In(Lu).[4] Several other red cell antigens are affected by In(Lu) and are all downregulated; these include Lutheran, P1, i, and mAb defined determinants of CD44. CD75 is readily detectable on red cells, though highly variable in expression from one donor to another. Partial penetrance of In(Lu) might account for this quantitative polymorphism. The predominant red cell determinant of CD75 is poorly expressed on lymphocytes and the predominant T cell determinant is only weakly expressed on red cells, evidence again suggesting selective expression of different glycoforms.

CD75 has the hallmarks of a functionally important cell surface antigen: it is an adherence molecule[5] and a differentiation antigen.[2 6] If the apparent microheterogeneity of CD75, suggested by the results of antibody binding studies, is a reflection of differences in oligosaccharide structures, this might contribute significantly to the possible functional repertoire of the molecule. As an adherence molecule, composite glycoforms of CD75 might have correspondingly complex ligands on other molecules on other cells. Alternatively, different glycoforms of CD75 might represent functionally active and quiescent adherence states. This may suggest an evolutionary strategy for functional diversification of leucocyte cell surface glycoproteins based on elaboration of carbohydrate determinants. Many other leucocyte molecules also carry complex and functionally active oligosaccharide structures. The fucosylated CD15 carbohydrate antigen (X determinant)—a product of the Lewis blood group locus—is specifically involved in cellular adhesion.[7] The CD15/X determinant is modified by the action of sialyltransferases: an α-2,3-sialyltransferase is essential for the formation of the sialyl Lewis X adherence determinant.[7] In myeloblasts, competition between α-2,3-sialyltransferase and the Lewis fucosyltransferase for the common substrate *N*-acetyllactosamine results in reduced expression of the X determinant.[8] The molecular basis of glycosyltransferase expression, in relation to the formation and expression of oligosaccharide blood group-like determinants on CD antigens on leucocytes, will be important in determining how possible structural and functional diversity might be achieved, and how the immune system would be influenced by polymorphism and mutations in enzymes and acceptors.

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Characterisation of the long and short forms of β1,4-galactosyltransferase

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β1,4-Galactosyltransferase (GalTase) has traditionally been considered a constituent of the Golgi complex, where it participates in the biosynthesis of oligosaccharides on glycoconjugates. GalTase is also present, however, on the cell surface of most cells, where it mediates cell-cell and cell-matrix interactions by binding to its glycoprotein substrate on opposing cells or in the extracellular matrix, respectively. The gene for GalTase has been cloned by a number of laboratories, and the sequence shows several potentially interesting features that may relate to the differential distribution of GalTase on the cell surface and in the Golgi complex. The cDNA contains two in-frame translation initiation start sites that result in translation of two nearly identical proteins, except that one contains an additional 13 amino acids at its cytoplasmically oriented N-terminus. To determine if these N-terminal differences play a part in the intracellular targeting of GalTase, RNA from cells that had characteristically different proportions of Golgi complex and cell surface GalTase activity was analysed by S1 nuclease protection assay. The results showed a strong correlation between the expression of the mRNA encoding the long form of GalTase and cell surface activity, and the mRNA encoding the short form of GalTase and Golgi complex activity. Transfection of the cDNAs for either the short or the long forms of GalTase into F9 embryonal carcinoma cells resulted in a three to fourfold increase in total GalTase activity in the transfected cells. Overexpression of the long form of GalTase, however, also resulted in a preferential increase in GalTase activity at the cell surface, compared with the short form, as assessed by subcellular fractionation of purified plasma membrane fractions.

These results suggested that the long form of GalTase may be the biologically relevant form of GalTase at the cell surface. To test this hypothesis more directly, truncated forms of either the short or the long forms of GalTase cDNA encoding the cytoplasmic and transmembrane domains were transfected into F9 and NIH 3T3 cells under the control of constitutive and inducible promoters. In all cases only cells transfected with the long truncated GalTase cDNA showed dramatic changes in adhesion, whereas those transfected with the short truncated cDNA were unaffected. The defect in adhesion was due to a decreased association of the long form of GalTase with the cytoskeleton as a result of competition between the truncated GalTase and the endogenous full length molecule for binding sites on the cytoskeleton.

The effects of overexpressing long and short GalTase on glycoprotein glycosylation were also examined in F9 cells as we wanted to determine whether any biological effects caused by perturbation of GalTase at the cell surface were due to altered glycosylation of cell surface glycoproteins. Total glycoproteins and a specific glycoprotein, LAMP-1, were examined in control cells and cells overexpressing either the long or short GalTase cDNAs. In all cases there were no significant differences in glycosylation between control and transfected cells. Control studies showed that the overexpressed GalTase was correctly localised in the transfected cells, and that GalTase substrates were in excess in all cells. Thus, in F9 cells, GalTase levels do not appear to be rate limiting during glycoprotein biosynthesis.

To assess the effects of altering the levels of cell surface GalTase on embryonic development and fertilisation, transgenic animals are being characterised that overexpress either the full length or truncated forms of GalTase.

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α1,3-Galactosyltransferase and autoimmunity

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Naturally occurring antibodies in human serum are constantly produced as a result of the exposure to normal environmental antigens such as gastrointestinal bacteria. Many of these antibodies seem to be directed against specific carbohydrate structures,[1 2] which requires a suppression of the synthesis of similar structures in human tissues. It has been proposed that the reaction between natural antibodies and a de novo synthesised forbidden carbohydrate antigen may be one mechanism by which autoimmune processes are initiated.[3]

The enzyme UDP–Gal:Galβ1→4GlcNAc α1→3-galactosyltransferase (α1,3-GT) transfers galactose to the terminal galactose residue of *N*-acetyllactosamine type carbohydrate chains and lactosaminoglycans according to the reaction:

$$\text{UDP–Gal} + \text{Gal}\beta1\rightarrow4\text{GlcNAc}\rightarrow\text{R} \xrightarrow{Mn^{2+}} \text{Gal}\alpha1\rightarrow3\text{Gal}\beta1\rightarrow4\text{GlcNAc}\rightarrow\text{R} + \text{UDP}$$

in which R may be a glycoprotein or a glycolipid.

The enzyme α1,3-GT exhibits a striking species-specific distribution: it is widely expressed in a variety of mammalian species, including the New World monkeys, but curiously no enzymatic activity is detected in Old World monkeys, apes, and man.[4] In addition, the serum of the latter species contains a natural IgG antibody (termed anti-αGal) that reacts with the enzymatic product with the structure Galα1,3-Gal.[5] Such antibody is absent from the serum of those mammals that do express α1,3-GT in their tissues.

A number of reports have linked α1,3-GT expression to the occurrence of diseases with autoimmune elements, such as Graves' disease, Chagas' disease, and systemic sclerosis.[6–8] These investigations are based on the detection of increased levels of anti-αGal. Whereas in parasitic diseases immunogenic Galα1,3Gal epitopes are present on the surface of the parasites, the increase in antibody titres as observed in specific autoimmune disorders is less readily explained, and has generated considerable interest. One theory is that the human genome might contain a functional α1,3-GT gene, but that transcription of this gene has been suppressed in the course of evolution. Under certain pathological conditions derepression would occur, which would result in an aberrant or de novo expression of α1,3-GT and the concomitant appearance of its product. Reaction of the 'forbidden' enzymatic product with the natural anti-αGal IgG might trigger or amplify an autoimmune process.[3]

With the long range goal of establishing whether the Galα1,3-Gal epitope has a role in human autoimmune disease we have started a study of the regulation of α1,3-GT activity in humans. We have isolated by molecular cloning a cDNA specific for bovine α1,3-GT.[9] This cDNA, used as a probe, did not detect α1,3-GT mRNAs in human cells or tissues, though α1,3-GT related sequences appeared to be present in the human genome. These observations suggest a suppression of α1,3-GT expression at the transcriptional level. Molecular cloning of the human genomic sequences by us[10] and others[11] indicated that the human genome contains two homologues of the α1,3-GT gene, located on the human chromosomes 9 and 12, respectively.[10] Both genes are pseudogenes. They contain several point mutations and frameshift mutations, and even if transcribed could not give rise to a functional protein. The homologue located on chromosome 12 lacks introns, and is organised as a retroposon. It might have originated about 30 million years ago. The second homologue, found on chromosome 9, is a pseudogene, in which the 'coding' region is interrupted by at least two introns. This gene most likely is the inactivated remnant of a once functional human α1,3-GT gene.

These observations argue against a role for α1,3-GT in disorders such as Graves' disease. What then is the explanation for the increase in anti-αGal titres in this disease? So far, no data are available on the precise structure of the human tissue epitopes that are the target for the anti-αGal IgG. It cannot be excluded that α-galactosylated structures other than Galα1,3Gal, if exposed on human tissues, might in fact be the preferred target of antiαGal IgG. Alternatively, if increased titres of anti-αGal are the result of a hyper-immunisation owing to the exposure of Galα1,3Gal epitopes on human tissues, these epitopes may be the product of a promiscuous α-galactosyltransferase, different from α1,3-GT but possibly related in primary sequence. The existence of such an enzyme is not likely in view of the evidence for the existence of a family of genes related to α1,3-GT.[10–13]

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Genes and IgG glycosylation in autoimmune and non-autoimmune strains of mice

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The oligosaccharide moieties of glycoproteins and glycolipids show a high degree of heterogeneity, with at least 30 different glycoforms of complex-type biantennary carbohydrate structures being found on human IgG.[1] Only a single copy gene (with two initiation sites) encoding uridine 5'-diphosphate (UDP)-galactose: *N*-acetylglucosamine β1, 4 galactosyltransferase (β1, 4 GalTase) has been found,[2] however, suggesting that all glycoproteins containing a β1, 4 *N*-acetylglucosamine-galactose (GlcNAc-Gal) linkage in their carbohydrate may be galactosylated using the same enzyme. Any control over the microheterogeneity of β1,4 galactosylation in oligosaccharides with exposed GlcNAc sugars might therefore depend on post-translational events, possibly including the action of putative regulatory subunits of the enzyme.

Abnormally reduced concentrations of IgG galactose are found in patients with rheumatoid arthritis (RA)[1] and in the arthritis prone MRL-lpr/lpr mouse.[3] These disease states also show decreased β1,4 GalTase activity and therefore provide a useful model for examining the control of galactosylation of a glycoprotein.[4 5] The β1,4 GalTase enzyme may be regulated by a 58 kilodalton GT-associated (p58GTA) protein kinase.[6] To determine if the β1,4 GalTase and p58GTA genes show polymorphisms which might lead to alterations in protein structure or dysregulation of gene expression, we carried out restriction fragment length polymorphism (RFLP) analyses of these genes.

We previously reported polymorphisms of the p58GTA gene in patients with RA and in control human subjects.[7] Using eight different restriction endonucleases, we have now carried out RFLP analyses of the genes encoding β1,4 GalTase and the p58GTA kinase, and determined IgG galactose concentrations, in a total of 11 autoimmune prone and non-autoimmune strains of mice. So far we have been unable to detect any polymorphism of the murine β1,4 GalTase gene. In both the non-obese diabetic (NOD) mouse, an animal model of human insulin dependent diabetes mellitus, and in the SJL mouse, which is susceptible to induced autoimmunity, however, all eight enzymes showed an alteration of the p58GTA gene structure. The figure (overleaf) shows a Southern blot of Hinc II digested liver DNA from eight of the mouse strains hybridised with a murine p58GTA cDNA. In contrast with NOD and SJL, the other mouse strains examined in this study (MRL-lpr/lpr, MRL−+/+, CBA/Ca, NZW, NZB, C57Bl, A/J, AKR, and DBA/2) gave identical patterns (for a given enzyme) with each of the eight enzymes tested. The NOD and MRL-lpr/lpr strain mice, but not the SJL strain mice, showed decreased concentrations of IgG galactose (figure; % Gal(0) represents the percentages of oligosaccharide chains without galactose residues). The degree of galactosylation of IgG in the SJL strain mice suggests that the observed polymorphism of the p58GTA gene in these mice does not negatively affect IgG glycosylation.

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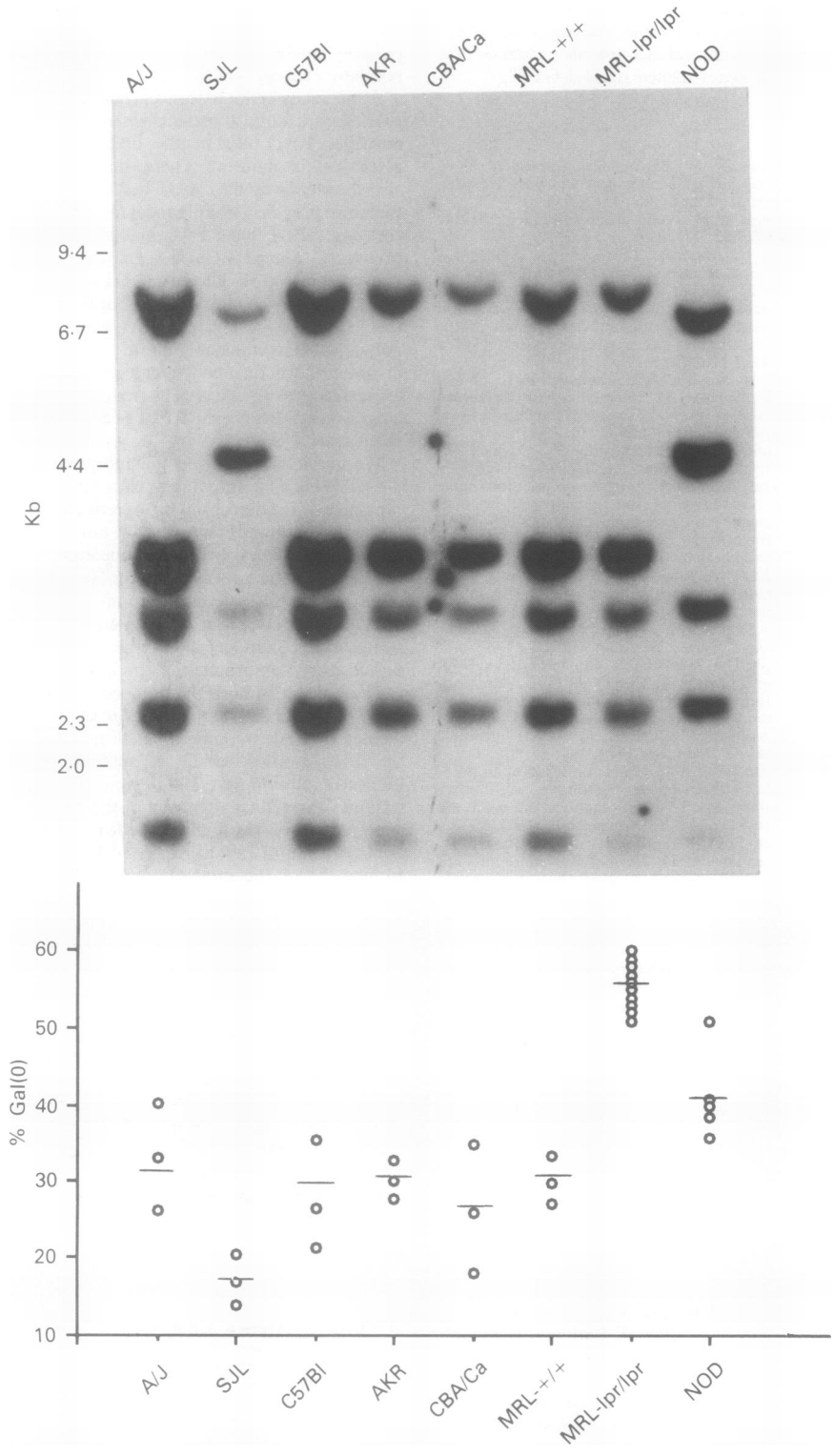

Southern blot of Hinc II digested liver DNA from eight mouse strains hybridised with a murine p58GTA cDNA.

Glycosylation networks in rheumatic disease

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Oligosaccharides are important in normal immune mechanisms and their relevance to autoimmune disease has recently come under scrutiny.[1] Data are presented to indicate why glycosylation changes may be associated with pathogenic mechanisms and a hypothesis is suggested, whereby an integrated glycosylation system is involved in normal immune mechanisms that may become dysregulated in disease. Manipulation of this system may offer significant potential in the development of new therapeutic strategies for the treatment of rheumatoid arthritis (RA) and other autoimmune rheumatic diseases.

DATA

Lymphocytic GalTase activity and serum IgG galactosylation

The synthetic abnormality associating reduced lymphocytic β1,4-galactosyltransferase (GalTase) and increased agalactosylated IgG (Gal(0)) with RA is now well documented.[1] To investigate potential mechanisms controlling glycosylation we studied the interrelationship between lymphocytic GalTase activity and Gal(0). We found that a positive feedback homeostatic mechanism regulating Gal(0) is present in the normal population, which is absent in those with RA, in whom there is a negative interrelationship.[2] This suggests that the synthetic abnormality in lymphocytic galactosylation might be a result of disruption of normal glycosylation control mechanisms.

Lymphocytic sialytransferase activity is also reduced in rheumatoid arthritis

We measured lymphocytic sialytransferase (STase) activity in RA to determine whether there are other glycosylation defects in this disease. A significant decrease (−41%) in STase activity was found compared with controls, and drug treatment and sex of the patient did not affect enzyme activity. Carbohydrate changes in RA may therefore be more widespread than previously envisaged.

Glycosyltransferase changes may occur in a variety of locations and arthritic disease

To determine whether glycosylation changes associated with arthritis are restricted to lymphocytes we measured serum STase and GalTase activities in RA, ankylosing spondylitis (AS), systemic lupus erythematosus (SLE), and osteoarthritis (OA).

STase activity was raised in all the diseases tested (18–35%, $p<0{\cdot}01$) compared with healthy subjects, whereas GalTase activity was only raised in AS (42·0%, $p<0{\cdot}001$) and OA (17·8%, $p=0{\cdot}038$). No correlation was found between STase and GalTase activity.

Thus increased protein glycosylation may occur in association with a variety of arthritic conditions, and glycosylation changes are therefore not restricted to lymphocytes.

What is the cause of reduced GalTase activity?

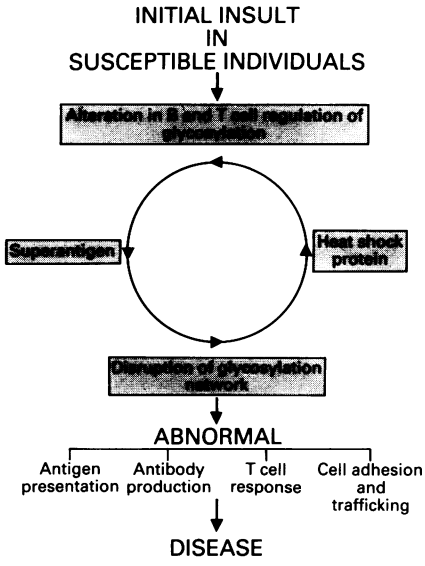
No evidence has been found to suggest the presence of an intracellular enzyme inhibitor nor are there gross structural changes in the GalTase activator gene.[3] Abnormalities in the expression of the GalTase gene may, however, occur and an antibody associated control mechanism has been sought.[4]

The presence of antibodies to GalTase has been shown in a variety of diseases. IgG GalTase antibodies were found to be significantly raised in RA, Crohn's disease, SLE, and pulmonary tuberculosis, but IgA antibodies were only raised in RA and Crohn's disease when comparisons were made with healthy subjects. IgM GalTase antibody levels, in contrast, were significantly decreased in all disease groups. IgG GalTase antibodies have subsequently been shown to correlate negatively with lymphocytic GalTase activity in RA. These data suggest that IgM antibodies may be part of a normal glycosylation control mechanism, whereas IgG antibodies are associated with disease and cause down regulation of L. GalTase activity.

CONCLUSION

1 Gal(0), GalTase, and GalTase antibodies are interrelated and may be part of a glycosylation control mechanism.

2 In RA there is likely to be both a defect in sialylation and galactosylation of B and T lymphocyte associated proteins. Also cell surface expression of STase (ie, CD75) and GalTase may be reduced, which may alter cell adhesion and disrupt lymphocyte trafficking.



Glycosylation networks in rheumatic disease.

3 Glycosylation changes may occur in other rheumatic diseases, and there may be an association between arthritis and distinct protein glycosylation patterns.

HYPOTHESIS:GLYCOSYLATION NETWORKS IN RHEUMATIC DISEASE

Our work has focused upon the interrelationship between IgG galactose, lymphocytic GalTase, and GalTase antibodies. This interrelationship may be part of a glycosylation network responsible for varying the carbohydrate content of proteins, and hence altering their function in response to feedback homeostatic mechanisms. Disease (for example, *Mycobacterium tuberculosis* or *Borrelia burgdorferi* infection) may lead to disruption of glycosylation control, resulting in aberrant immune function. In certain subjects this may become self perpetuating and result in arthritis as well as other reactive conditions (figure).

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Inflammation

Increased expression of a lysosomal membrane glycoprotein in the cell surface of poorly metastatic colonic cells results in adhesion to E-selectin expressing cells

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The lysosomal membrane glycoproteins, lamp-1 and lamp-2, are the major carriers for poly-*N*-acetyllactosamines.[1] We have shown that highly metastatic colonic tumour cell lines contain more poly-*N*-acetyllactosamines in carbohydrates attached to lamp molecules than poorly metastatic cell lines derived from the same human colon carcinoma. The studies also showed that poorly metastatic cell lines express fewer lamp molecules on the cell surface than highly metastatic sublines.[2]

As lamp molecules probably carry carbohydrate ligands for E- and P-selectin, we studied whether the increased expression of lamp-1 on the cell surface results in stronger adhesion to cells expressing E-selectin. Poorly metastatic SP cells were transfected with cDNA encoding wild type lamp-1 or lamp-1 mutant in which the cytoplasmic tyrosine was substituted with histidine. As shown previously, mutation of cytoplasmic tyrosine abolishes lysosomal targeting of lamp-1, and mutated lamp-1 is transported to the plasma membrane instead of going to lysosomes.[3]

Several clones were isolated from each transfection and representative cell lines were cloned, which show different amounts of cell surface lamps by flow cytometric analysis. In parallel, Chinese hamster ovary (CHO) cells were transfected to express E-selectin stably. The adhesion of various SP cells expressing different amounts of cell surface lamp-1 was tested for the adhesion to E-selectin expressing CHO cells, as well as interleukin 1β activated endothelial cells.

The results clearly indicate that the extent of the cell adhesion is directly proportional to the amount of cell surface lamp. This adhesion can be inhibited by sialyl Le^x containing glycolipid, whereas control sialyl paraglogoside had no effect. Such adhesion can be inhibited by the culture medium derived from A431 cells continuously expressing soluble lamp-1, and synthesising sialyl Le^x structures.

These results indicate that the increased surface expression of lamp-1 results in better adhesion to cells expressing E-selectin. The results suggest that these tumour cells probably adhere to endothelial cells at metastatic cells through the interaction between cell surface carbohydrates of tumour cells and E- and P-selectins on endothelial cells. Further studies are necessary to determine if the increased expression of lamp-1 on the cell surface results in tumour metastasis.

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A modified function for agalactosylated IgG?

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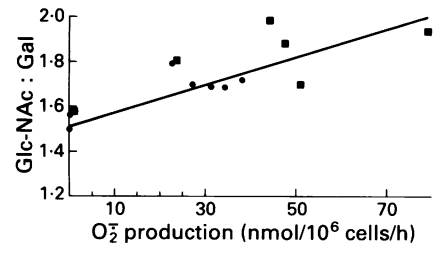
Immunoglobulins are multifunctional proteins whose primary role is in the binding and clearance of antigens, either via receptor mediated uptake or activation of complement. On IgG, specific amino acids, or sequences thereof, have been identified as critical for the recognition of antigen, Fc receptors, and complement.

As a glycoprotein, IgG has two oligosaccharide moieties added postsynthetically to each H chain at Asn-297, with a further 30% incidence of carbohydrate residues in the variable regions.[1] The H chain bound carbohydrate moiety maintains the structural integrity of the CH2 domain and has been shown to be a requirement for efficient feedback suppression in mice.[2] There is also evidence to suggest that the oligosaccharide may play a significant part in the turnover of immunoglobulins in the circulation.[3] These findings indicate that oligosaccharides are fundamental to the recognition of IgG, a hypothesis also supported by the observation that asialylated IgG is immunogenic in autologous hosts.[4]

Studies on the carbohydrate composition of circulating IgG have shown a reduction of terminal galactose (Gal) in several inflammatory diseases, most notably rheumatoid arthritis (RA), thus exposing *N*-acetylglucosamine (Glc-NAc) as the terminal residue.[5] Based on these findings it has been postulated that, in RA, agalactosylated IgG may be antigenic and be a stimulus for rheumatoid factor production.[6] No relation has been found, however, between the degree of agalactosylation on IgG and rheumatoid factor reactivity.[7]

As a multifunctional protein, any modifications to the structure of IgG might be expected to have diverse effects. Therefore, we investigated the ability of IgG to activate resting polymorphonuclear leucocytes (PMNs), and related our findings to the degree of agalactosylation on IgG. Briefly, IgG was isolated from the serum and synovial fluid of rheumatoid patients according to the method of McKinney and Parkinson,[8] with further purification by molecular weight, using gel filtration high performance liquid chromatography to produce monomeric IgG. PMNs were isolated from the peripheral blood of normal healthy volunteers by density gradient centrifugation, and after resting at 37°C for 15 minutes they were exposed to isolated IgG. PMN activation was measured as superoxide production (O_2^-), and followed spectrophotometrically as the superoxide dismutase inhibitable reduction of cytochrome *c*.[9] The glycosylation status of IgG was determined by a modified enzyme linked immunosorbent assay (ELISA) using lectins of defined specificity.[10]

The figure shows that a direct linear correlation exists between Glc-NAc:Gal and O_2^- production, and that synovial fluid IgG was always a more effective stimulus for O_2^- production than the corresponding paired



Linear regression analysis showing the direct relation between the glycosylation status of IgG and its ability to generate O_2^- radicals from resting polymorphonuclear leucocytes, where IgG was isolated from the serum (●) or synovial fluid (■) of patients with rheumatoid arthritis. The correlation coefficient has been calculated as $r=0{\cdot}753$, for 13 data points, and $p<0{\cdot}01$.

serum. It not clear from these results whether IgG is reacting with Fc receptors present on PMNs or whether activation is being achieved by some other process, such as the cell surface Glc-NAc receptors normally involved in bacterial binding.

These data show that changes to the carbohydrate moiety on IgG are associated with a modification in function, and this may be predicted to have important effects in vivo; at an inflammatory site the agalactosylation of IgG may play a part in further phagocytic cell activation and, by the generation of the superoxide anion, may be important in maintaining the chronicity of inflammation observed in the rheumatoid joint.

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Cytokines and glycosylation

Control of interferon γ glycosylation: how sweet is the sugar coated pill?

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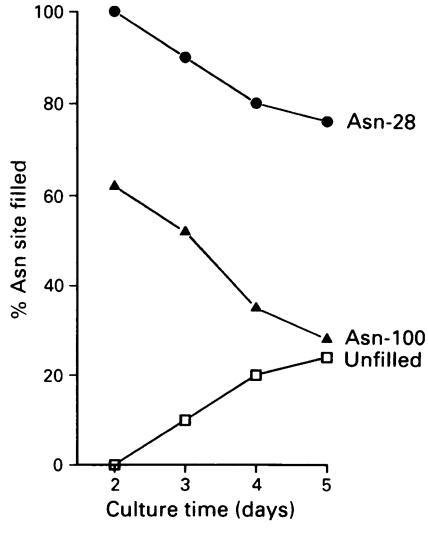
Human interferon γ (HuIFN γ) is a lymphokine normally secreted by antigen sensitised T lymphocytes, which stimulates major histocompatibility complex class II expression and which possesses potent antiviral, antichlamydial, and antitumour properties. Natural and recombinant Chinese hamster ovary (CHO) derived IFN γ is glycosylated with complex biantennary N-linked oligosaccharides attached to one or both of the available asparagine (Asn) sites present, yielding a mixture of fully and partially glycosylated IFN γ molecules. When recombinant IFN γ was produced by CHO cells in batch culture, however, up to 12 molecular weight variants were secreted, which were reduced to three when the cells were pretreated with tunicamycin, an antibiotic which blocks glycosylation. Treatment with peptide:N-glycosidase F, an enzyme which specifically removes N-linked oligosaccharides, yielded the same three molecular weight forms, suggesting that the IFN γ molecule was variably processed by an intracellular protease.[1] The post-translational modifications of variable Asn site occupation and proteolytic processing of the C terminus were thus responsible for the generation of multiple IFN variants produced by CHO cells.

The degree of oligosaccharide substitution at each Asn site also varied with time in batch culture; a major shift to underglycosylation of the IFN γ polypeptide was seen in small and large scale cultures. This reduced the level of oligosaccharide substitution at both positions steadily, from 50 to 60 hours of culture onwards. After five days of culture the proportion of Asn-100 sites filled was up to 50% less than the proportion of Asn-28 sites occupied on the IFN γ polypeptide (see figure). Evidence from *x* ray crystallography of the IFN γ dimer suggests that protein folding constraints may be affecting the efficiency of the glycosylation process at Asn-100, as it forms part of an α helical structure in the folded molecule.

As the gene encoding HuIFN γ has been cloned and sequenced,[2] the technique of site directed mutagenesis was then used to convert either Asn-28 or Asn-100 into glutamine residues at the DNA level, to prevent glycosylation at either the first or the second site. The mutated IFN cDNA was subcloned into the pSVL mammalian expression vector and then transfected into COS-7 cells. When the gene products were analysed by immunoprecipitation of ^{35}S methionine labelled material followed by sodium dodecylsulphate polyacrylamine gel electrophoresis and autoradiography, there was a 100% increase in Asn-100 site occupation when the Asn-28 site had been removed, compared with controls. In contrast, there was only a 10% improvement in Asn-28 site occupation after Asn-100 mutagenesis.

In summary, the observed variability in the post-translational modification of IFN γ by CHO cells is thought to be due to (*a*) competition between glycosylation sites (possibly for glycosyltransferase enzymes or their substrates, or both), (*b*) protein folding (secondary structure), (*c*) carboxyterminal processing of the polypeptide, and (*d*) culture environment induced effects on CHO cell physiology. Attempts to reduce the level of recombinant IFN γ heterogeneity are currently in progress.



Glycosylation of interferon γ by Chinese hamster assay cells in batch culture. (▲) Asn-100; (●) Asn-28; and (□) unfilled site.

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Agalactosyl IgG, interleukin 6, and pregnancy

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It is likely for three reasons that interleukin 6 (IL-6) plays a part in the regulation of agalactosyl IgG (Gal(0)): (*a*) levels of Gal(0) are extremely high in transgenic mice overexpressing the human IL-6 gene[1]; (*b*) direct injection of recombinant IL-6 into normal DBA/1 mice results in a rise in serum agalactosyl IgG[2]; and (*c*) IL-6 causes changes in the activity of glycosyltransferases, including galactosyltransferase, in B cells.[3]

There is also circumstantial evidence: (*a*) levels of IL-6 in the peritoneal cavity correlate with the percentage agalactosyl IgG (% Gal(0)) in mice given intraperitoneal injections of mineral oil (pristane arthritis model)[2]; (*b*) Gal(0) and IL-6 are simultaneously raised in patients with Castleman's disease or IL-6-secreting tumours[4] (and our unpublished observations); and (*c*) the most strikingly raised cytokine in rheumatic joints is IL-6, which seems to be released spontaneously in vitro by cells derived from such joints.[5]

Interleukin 6 is unlikely to be the only regulator of Gal(0). A study of serum samples from pregnant mice and humans indicated that the %Gal(0) may also be regulated by endocrinological factors.[6] Thus the % Gal(0) falls to very low levels during pregnancy in mice and humans, and rises again rapidly in the postpartum period. The fall occurs even in pregnant patients with rheumatoid arthritis, where the changes seen in pregnancy are similar to those seen in pregnant normal women, except that all the values are higher. This suggests that the mechanisms which lower the levels in pregnancy are distinct from the mechanisms which set the level too high in rheumatoid arthritis. The raised %Gal(0) which accompanies collagen II arthritis,[6] or pristane arthritis in mice, also falls in pregnancy.[6 7]

Because the maternal %Gal(0) is so low throughout the latter half of pregnancy the level is also very low in neonates. It stays low in the neonate until three months when it suddenly rises, reaching a high adult level by six months (Pilkington *et al*, unpublished

data). We therefore wondered whether this pattern was different in babies with autoimmune disorders. Collaborative studies of neonatal lupus (with Pamela Taylor, Leeds) and neonatal *Myasthenia gravis* (with Ann Kari Lefvert, Stockholm) have led to the observation that the appearance of neonatal symptoms in babies of afflicted mothers correlates with the %Gal(0) in the maternal and neonatal circulations at term, rather than with the titre of the relevant autoantibody. These observations lead to several interesting speculations about the regulatory and effector properties of agalactosyl IgG, as reviewed elsewhere.[8 9]

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Role of the Fc-N-glycosylation of IgG in the production of tumour necrosis factor α induced by the cross linking of human monocyte Fcγ receptors

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Serial lectin chromatography of native, normally glycosylated IgG has confirmed that there exists within the total IgG population a range of affinities for monocyte Fcγ receptors (FcγR) which is independent of subclass, but correlates with Fc-N-glycosylation. Further, this Fc-N-glycosylation is mainly involved in the post-binding events as the engulfment step occurring after the FcγR-IgG interaction. A rapid secretion of tumour necrosis factor (TNF-α) in response to the cross linking of human monocyte FcγR by IgG coated red blood cells (IgG RBCs) is another Fc dependent post-binding event.

To study the eventual role of the Fc-N-glycosylation on this production, anti-RBC rabbit IgG antibodies differing from each other by their capacity to bind to lectins were prepared by serial affinity chromatography on lectin coated columns. Four different preparations of the same anti-RBC rabbit IgG antibody batch (glycoforms) were coated to RBCs; the first one was the starting antibody preparation (IgG-total) and the three others were IgG antibody preparations with preferential binding capacities to peanut agglutinin (IgG-PNA) (recognising accessible β-galactosyl residues), to concanavalin A (IgG-Con A) (recognising accessible α-mannosyl residues), or to *Bandeiraea simplicifolia* BS-II (IgG-BS-II) (recognising accessible *N*-acetyl-β-glucosaminosyl residues). They showed the same haemagglutinating antibody titres. TNF-α concentrations were determined in the supernatants of the cell cultures with a TNF-α specific IRMA kit (lower detection limit 5 pg/ml) (Medgenix; Belgium). Five normal subjects were studied.

TNF-α concentrations <0·3 ng/ml were seen when monocytes were incubated for three hours at 37°C with: (*a*) the culture medium alone; (*b*) soluble anti-RBC IgG-total (2·5 mg/ml); (*c*) RBCs alone; (*d*) RBCs coated with $F(ab')_2$ fragments of the anti-RBC IgG-total antibody preparation. On the contrary, monocytes incubated with RBCs coated with 2000 anti-RBC IgG-total/cell produced TNF-α concentrations ranging from 1 to 3 ng/ml, independently of the presence or not of polymyxin B (10 μg/ml). When monocytes were incubated with RBCs coated with 2000 IgG-BS-II anti-RBC IgG/cell, TNF-α concentrations rose to 5–10 ng/ml, whereas TNF-α concentrations obtained in the presence of RBCs coated with the same number of IgG-PNA or of IgG-Con A did not significantly differ from those seen in the presence of RBCs coated with IgG-total. This effect was independent of a prior exposure of monocytes to solutions of: (*a*) trypsin (0·025%); (*b*) cytochalasin B (0·02 mol/l); (*c*) various carbohydrates of the Fc domain (10–50 mmol/l).

These data suggest that the Fc-N-glycosylation of IgG is involved in the TNF-α production induced by the cross linking of human monocyte FcγR. Further, as the most potent inducers of TNF-α production were IgG glycoforms with mainly terminal or accessible *N*-acetyl-β-glucosaminosyl residues, or both, they may be of pathogenetic relevance in rheumatoid arthritis, a disease characterised by a deficient Fc-N-glycosylation of IgG.

Clinical observations and application

Abnormalities in the glycosylation of IgG: clinical aspects

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It was established in 1985 that the percentage incidence of agalactosyl structures (with both outer arms terminating in *N*-acetylglucosamine on serum IgG) in patients with rheumatoid arthritis (RA) was well above average compared with a group of normal subjects.[1] At that time is was thought that patients with osteoarthritis occupied an intermediate position with respect to the frequency of agalactosyl IgG chains. A study of 151 healthy subjects of both sexes, varying in age from 1 to 70 years, however, showed that the relative incidence of agalactosyl N-linked oligosaccharides on total serum IgG (Gal(0)) was related to age.[2] In particular, it was noted that the Gal(0) concentration decreased from 1 year of age to 15 years of age. It then remained almost constant up to the age of 40 years but then it steadily rose with increasing age. Thus the patients with osteoarthritis did in fact fall within the normal range.

In a large study of approximately 25 different diseases it was noted that increased concentrations of Gal(0) were essentially confined to patients with RA, pulmonary tuberculosis, Crohn's disease, and some patients with lupus complicated by Sjögren's syndrome.[3] It was noted in particular that patients with viral infections, including AIDS, bacterial infections, other autoimmune rheumatic diseases, diabetes, rheumatic fever, and osteoarthritis had normal concentrations. Subsequently, it has been shown that patients with leprosy, although usually having normal Gal(0) concentrations, have increased concentrations in the presence of an attack of erythema nodosum leprosum.[4] In serial studies of children with juvenile chronic arthritis it was shown that the concentration of Gal(0) reflected the level of disease activity[5] and similarly it has been shown that successful treatment of patients with tuberculosis is mirrored by decreases in the Gal(0) concentration (Isenberg, Parekh, Bottonley, Rademacher, unpublished observations).

Approximately 75% of patients with RA who become pregnant go into remission. In a study of pregnant women with RA we showed that those who go into remission have Gal(0) concentrations that decrease significantly.[6] Intriguingly, healthy subjects who are pregnant also have a reduction in their pre-pregnant Gal(0) level. Other data have suggested that there may be a selective transfer of normally galactosylated IgG1 across the placenta. In contrast, IgG2, IgG3, and IgG4 are not actively transported across the placenta. It was also established that Gal(0) concentrations in healthy children increase rapidly during the first year of life to between 30 and 50%, suggesting that when the neonatal immune system begins to manufacture its own immunoglobulins many of the IgG molecules produced are agalactosylated. In more recent studies we have sought to determine whether an increased Gal(0) concentration is present early in the course of adult onset RA. In a study of 60 patients presenting to two early synovitis clinics, it was shown after two years' follow up that 39 patients had indeed developed RA and 21 had developed a variety of other disorders, including a parvovirus infection and lupus.[7] The positive predictive power of rheumatoid factor and Gal(0) separately were shown to be around 80%. In patients presenting with early synovitis who had a positive rheumatoid factor *and* an increased Gal(0), however, a positive predictive value of 94% was recorded. Furthermore, in an intriguing study of Pima Indians, who appear to be genetically predisposed to developing RA, it has been shown that the mean galactose content of the serum IgG from 11 rheumatoid patients collected before disease onset was significantly lower than IgG from seronegative subjects without the disease from the same group.[8] Taken together, these observations imply that the defect is present not only early in the course of RA, but may even antedate its onset.

In a family study of 31 family members and

spouses of eight patients with RA (supplemented by a further 13 patients and spouses) it was shown that the glycosylation defect was present in the relatives of the RA probands who also had the disease and, intriguingly, in 13 of 21 spouses of the patients, but rarely (four of 26) in their healthy first degree relatives.[9] These data strongly suggest that an environmental factor must be playing a key part in the development of the impaired glycosylation.

In studies undertaken by Breedveld and Rook (unpublished observations) it has been shown that the Gal(0) at the onset of disease in patients with RA is correlated with a number of outcome features assessed during a follow up period of eight years. Thus the age corrected Gal(0) concentrations correlated statistically significantly with the number of swollen joints, the Health Assessment Questionnaire score, erosion score, and the Steinbrocker functional test.

In a collaborative ongoing study between the department of rheumatology at St Alban's City Hospital and Bloomsbury Rheumatology Unit we are attempting to determine the relation between the Gal(0) concentration and disease activity in patients with RA to determine whether any particular drug is better able to correct the Gal(0) concentration, and to confirm the observations of Breedveld and Rook. In summary, measuring Gal(0) concentrations has been shown to be of clinical significance in terms of disease specificity, in reflecting disease activity in juvenile chronic arthritis, and in offering clues to the aetiopathogenesis of this major debilitating disease.

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Mannose binding protein and infection

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Human mannose binding protein (syn. mannan binding protein, MBP, core specific lectin) is a calcium dependent (C-type) lectin secreted by the liver and with specificity for mannose and glycoproteins terminating in *N*-acetylglucosamine. The protein occurs as multimers of an identical peptide chain of 32 kilodaltons having the following three distinct regions: (*a*) a cysteine rich N-terminal region involved in interchain disulphide bonding, (*b*) a region of Gly-Xaa-Yaa repeats typical of collagenous proteins, and (*c*) a C-terminal region containing some 18 invariant amino acid residues characteristic of all C-type animal lectins. Three of the chains associate through disulphide bonding and the formation of a collagenous triple helix. The resultant 96 kilodalton subunit polymerises to give a range of molecular forms ranging from trimers and tetramers to pentamers and hexamers.

Mannose binding protein is believed to have a major role in host defence against pathogens with mannose rich cell walls. Firstly, it resembles IgG and IgA in that it is able to act directly as an opsonin, and, after binding to a microbial surface, can interact with the C1q receptor of phagocytic cells. Secondly, MBP is able to activate the classical pathway of complement through an antibody independent mechanism involving the binding of $C1r_2$-s_2 complexes to surface bound MBP.

In the last three years a link has been established between low serum concentrations of MBP and a common opsonic deficiency (manifested as a failure to opsonise bakers' yeast) first described in an infant with severe recurrent infections, failure to thrive, and diarrhoea. Subsequently, the yeast opsonisation defect was identified in 11 of 43 children with frequent unexplained infections, in association with chronic diarrhoea of infancy and in a significant proportion of children developing otitis media during the first year of life. In addition to these clinical associations, several groups have reported that the defect is relatively common (incidence about 5–7%) in various apparently healthy populations. It has been established that at the concentration of serum used in these assays the level of MBP in a serum regulates the deposition of C4 and C3 fragments. Those subjects with a profoundly low level of MBP manifest poor functional activity in the yeast opsonisation assays. The link between low levels of MBP and the common opsonic defect was confirmed by dose dependent in vitro correction of the functional defect using purified MBP.

The genetic basis of MBP deficiency is now becoming clear. Studies in three British families, each with a child having a history of recurrent infections and low serum concentrations of MBP, have shown evidence for a point mutation in codon 54 of the MBP gene. This codon (GGC) is located in exon 1 and encodes for part of the collagenous sequence. The mutation to GAC results in an amino acid change from glycine to aspartic acid and would be expected to distort the secondary structure of the triple helix profoundly as the repeating glycines are axial and aspartic acid has a side chain which would be difficult to accommodate within the helix. In a study of 16 family members the codon 54 mutation co-inherited with low levels of serum MBP in an autosomal dominant fashion, and all subjects with a low MBP concentration were either heterozygous or homozygous for the codon 54 mutation. It remains to be established by in vivo studies whether mutant MBP with disordered secondary structure is degraded intracellularly or after secretion.

Glycosylation of IgG in the collagen induced model of arthritis

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Biochemical differences have been noted on the IgG molecules in patients with rheumatoid arthritis. Oligosaccharides on the Fc region of IgG usually terminate in either sialic acid or galactose, but patients with rheumatoid arthritis often lack terminal galactose and thus expose an *N*-acetylglucosamine.

We examined IgG molecules from the collagen induced model of arthritis for glycosylation changes. The collagen induced arthritis in DBA/1 mice is considered to be a good animal model for rheumatoid arthritis.

Glycoforms of IgG were detected by immunoblotting with a panel of lectins. Galactose was readily detectable with *Ricinus communis* agglutinin on DBA/1 mouse IgG. The galactose was significantly lower on the IgG samples from the collagen induced arthritic group ($p=0{\cdot}027$) than on samples from unimmunised controls matched for age. *N*-Acetylglucosamine as detected by *Bandeiraea simplicifolia* was significantly raised on the IgG samples from the group with collagen induced arthritis compared with the age matched, unimmunised control group ($p=0{\cdot}0001$). Sialic acid as measured by *Sambucus nigra* was readily detectable on DBA/1 mouse IgG, but no significant difference was seen between the arthritic and control group.

Oligosaccharides are thought to play an important part in complex formation. To establish their role we investigated the oligosaccharide profiles of both serum IgG and complexed IgG on a 10% sodium dodecyl sulphate reducing gel. Sialic acid, galactose, and *N*-acetylglucosamine were all readily detected in both serum IgG and complexed IgG.

The similarities betwen the glycosylation changes of IgG in an experimental model of arthritis and in patients with rheumatoid arthritis are interesting. There may be common pathogenic mechanisms in experimental arthritis and rheumatoid arthritis.

Agalactosyl IgG (Gal(0)): report of its clinical application in early inflammatory arthritis

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High concentrations of agalactosyl IgG Gal(0) have been reported in adult and juvenile patients with rheumatoid arthritis (RA), particularly in those patients with active disease.[1] This report describes studies that have been performed to determine the clinical significance of Gal(0) concentrations in the early stages of adult onset inflammatory arthritis.

In one study of 60 consecutive patients from an early synovitis clinic, Gal(0) concentrations (corrected for age and sex) were significantly increased in 39 patients who developed RA compared with 21 patients who were eventually diagnosed as having other inflammatory arthropathies ($p<0{\cdot}0001$). A combination of increased Gal(0) and a rheumatoid factor (RF)

titre greater than 1/40 gave a positive predictive value for a diagnosis of RA in 94% of these patients.[2]

In further studies the potential use of Gal(0) as a marker of disease activity and its ability to predict the clinical outcome of patients with RA have been investigated. Gal(0) tests were peformed on 40 patients with RA at their first consultation and at regular (at least yearly) intervals over five years. Clinical assessments were recorded at the same time and included the duration of early morning stiffness, joint score, distribution of joint disease, number of American Rheumatism Association criteria, functional status (Health Assessment Questionnaire (HAQ)), visual analogue pain scale, grip strength, erythrocyte sedimentation rate (ESR), haemoglobin, platelet count, RF titre, *x* ray score, extra-articular features, type of drugs used, and course or pattern of disease.

The patient group consisted of 26 women, mean age 51·6 years and 14 men, mean age 53 years. All the patients were seen within two years of the onset of RA (76% within one year) and before the prescription of any second line slow acting drugs. Eighty per cent of the patients were RF positive, and 77% showed erosions on radiographs.

The mean Gal(0) at first visit was 0·88 × standard deviation (SD) above the age corrected mean and increased to 2·17 SD above the mean after four years' follow up. In contrast, most clinical and laboratory features of disease activity (expressed as mean values) improved with time over four years. If all available data were analysed, positive associations between Gal(0) concentrations and increasing age of onset of RA ($p<0·05$), HAQ score ($p=0·01$), and extra-articular features of RA, including nodules and vasculitis ($p=0·001$), were observed. Further analysis was confined to the first visit and four year samples in the same patients. Gal(0) correlated with the HAQ score and ESR at four years' follow up ($p<0·01$), but not at onset. The initial Gal(0) concentration on its own at onset did not predict clinical outcome at four years.

We have shown that Gal(0) concentrations differentiate between early RA and other inflammatory arthropathies, and in patients with RA Gal(0) is associated with certain measures of disease activity. Further studies are required to determine which combination of clinical and laboratory measures and Gal(0) concentrations at onset best predict the outcome of early RA.

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Effect of conformational changes in IgG Fc epitopes influenced by the carbohydrate side chain in the C-γ-2 domain on rheumatoid factor binding

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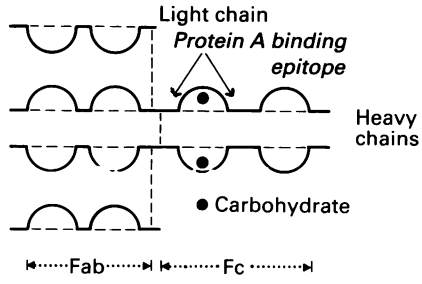
Changes in the nature of the carbohydrate located in the Fc portion of IgG (figure) have been reported to occur in patients with rheumatoid arthritis (RA), in patients with other chronic inflammatory diseases, and as a part of the normal process of aging. As rheumatoid factors (RFs) are thought to bind in or near the region where protein A binds, a region potentially influenced in conformation by the carbohydrate at position 297, we investigated the effect of the carbohydrate on RF binding.

Purified Fc fragments that varied in the type of carbohydrate present were transferred to nitrocellulose gels after separation by sodium dodecylsulphate polyacrylamide gel electrophoresis. Binding of human monoclonal RFs derived from patients with mixed cryoglobulinaemia, RA, systemic lupus erythematosus, or from normal subjects could be shown using antibodies specific to light chain immunoglobulins. Of the eight IgG RFs tested, maximum binding was detected to the Fc preparations that were fully glycosylated. Only one of seven IgG RFs derived from a normal subject was found to bind to the Fc region when the carbohydrate was removed by *N*-glycanase treatment (79% of the maximum binding).

As we had previously shown, by using an enzyme linked immunosorbent assay (ELISA), that IgM RFs bound equally well to all Fc preparations regardless of the nature of the carbohydrate on the Fc region, we speculated that perhaps the large pentameric nature of the IgM RF might compensate for changes in affinity brought about by changes in the carbohydrate. To test this hypothesis, Fab fragments of two monoclonal IgM RFs were used in the immunoblotting binding assay as described earlier. Only one Fab preparation (KAS) was found to bind to the Fc fragments, and at a 3·5 fold lower binding than with intact IgG RFs (two Fab arms). (Pentameric IgM RFs were found to bind 250 times higher than IgG RFs in the immunoblot assay.) Interestingly, maximum binding of the KAS Fab was to the fully glycosylated Fc fragments: 26% of the maximum binding was to Fc fragments which had been treated with *N*-glycanase and 47% of maximum binding was to Fc fragments which had a less accessible mannose (that is, which lacked the ability to bind to concanavalin A). For the IgG RFs, 3 and 1% binding was observed to the *N*-glycanase treated Fc and the Fc fragments with less accessible mannose respectively. The KAS Fab bound equally well to the polyclonal Fc, isolated from a patient with RA, which had been shown to have less galactose present than normal polyclonal Fc fragments. IgG RFs, on the other hand, had 16% of the maximum binding to the Fc fragments containing low amounts of galactose. No binding of any of the RFs tested could be detected to Fc preparations that were reduced to single polypeptide chains, indicating that a conformational epitope dependent on disulphide bonding was important. In addition, as the carbohydrate was still present on the single polypeptide chains and no binding was observed, it could be concluded that there was no direct binding to the carbohydrate itself. These studies indicate that the RF-Fc interaction is influenced by the nature of the carbohydrate on the Fc portion but to a variable degree, being greatest for IgG RFs. Further studies with additional Fab fragments of IgG and IgM RFs will determine the degree of variability. The explanation for the variability might lie in different antigen selection events occurring for IgG RF versus IgM RF secreting B cells.



Schematic representation of IgG1 showing the Fab and Fc portions of the molecule and the location of the carbohydrate side chain. The protein A binding epitope is delineated.

Altered regulation of glycosylation in malignant cells

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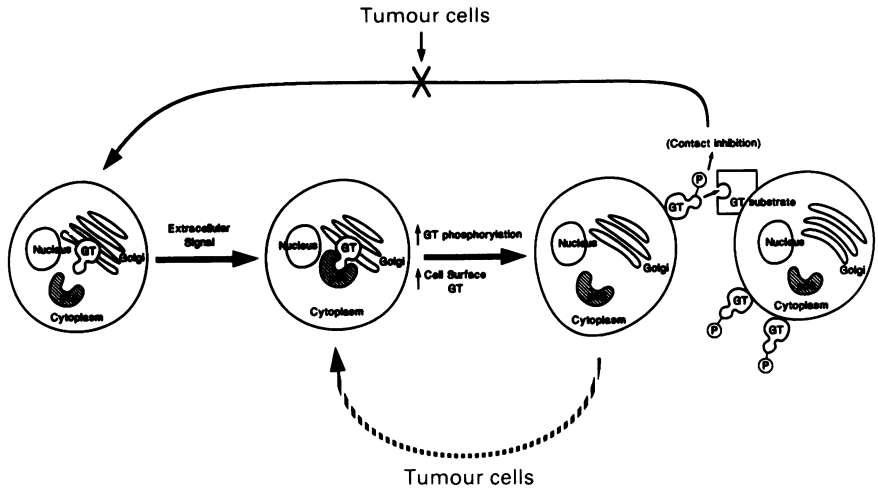
The role of cell surface glycoconjugates and glycosyltransferases in development, differentiation, and transformation has been studied for some time. Changes in carbohydrate moieties of glycoconjugates that occupy the cell surface have been noted in numerous transformed cells, but their importance has been obscure. Similarly, the appearance of Golgi enzymes that are involved in glycosylation events on the cell surface has been an enigma. Of the glycosyltransferases, the role of β1,4-galactosyltransferase (GalTase) has been studied most extensively with regard to these processes.

Cell surface GalTase has been shown to function in cell adhesion and cell proliferation. During fertilisation murine sperm cell surface GalTase and the egg-coat glycoprotein ZP3 function as complementary adhesion molecules that mediate gamete binding.[1] Changes in cell surface GalTase have also been observed during other cellular processes. For example, increased cell surface GalTase expression in murine L 1210 leukaemic cells has been shown to have a potential role in the proliferative capacity of these cells.[2] In yet other cell systems, such as the rat parotid gland, β-adrenergic agonist stimulation of these cells leads to a rapid topographical rearrangement of Golgi GalTase to the cell surface via an unknown mechanism(s).[3 4] These observations suggest the GalTase may also play a part in proliferation when localised on the cell surface.

Changes in GalTase gene expression do not account for the rapid increase in cell surface localised enzyme. One potential explanation is that GalTase is subject to rapid post-translational modification. It has been shown that GalTase is phosphorylated both in vitro and in vivo.[5 6] Dephosphorylation of the native enzyme seems to decrease its enzymatic activity, whereas increased phosphorylation of the enzyme leads to an apparent increase in its enzyme activity.[6] These phosphorylation events may be mediated by a specific protein kinase, p58, which was found to co-purify with the GalTase enzyme.[6 7] Detailed characterisation of the p58 protein kinase has shown that it is a member of the cell division, control related protein kinase gene family. Both



A hypothetical model for the possible association between the p58 protein kinase and β1,4-galactosyltransferase (GalTase). The model proposes that in response to extracellular signals GalTase might be phosphorylated and subsequently transported to the cell surface where it could interact with a suitable substrate on a cell in close proximity. This could then lead to contact inhibition of cell growth. Abrogations of this process, due to alterations in the protein kinase that phosphorylates GalTase or in GalTase itself, would lead to altered cell growth patterns and loss of contact inhibition.

GalTase and p58 gene expression are regulated in a cell cycle dependent manner; galactosyltransferase peaks during early G_1 and S phases and decreases during the G_2/M phase of the cell cycle, whereas p58 peaks during late G_1 and S phases and decreases during the G_2/M phase of the cell cycle, suggesting that p58 may be responsible for phosphorylation of GalTase (figure).[8] Furthermore, minimal alteration of the normal level of p58 gene expression in eukaryotic cells leads to gross abnormalities of cell cycle progression, cell morphology, chromosome ploidy, and cytokinesis.[7]

The biochemical interactions of these growth regulatory genes, in addition to their locations in genomic regions frequently targeted in neoplasia, may indicate that they have a concerted role in the development of certain cancers. The GalTase gene localises to the short arm of chromosome 9 (9p13–p21) whereas the p58 gene localises to the short arm of chromosome 1 (1p36).[9 10] One allele of the 1p36 region is frequently deleted in malignant melanoma, neuroblastoma, endocrine neoplasia, and certain forms of breast carcinoma.[10] Similarly, a tumour suppressor locus that undergoes mutation during the early stages of melanoma development has been localised to the 9p region.[11] Preliminary studies indicate that the GalTase locus is not affected in melanoma, but that the p58 locus is consistently deleted and translocated in neuroblastoma, and that expression of the p58 gene is reduced (unpublished results).[12] Further molecular analyses of the remaining p58 allele by reverse transcriptase-polymerase chain reaction are currently being carried out on samples from patients with neuroblastoma to examine the nature of the expressed p58 gene product. These studies may provide some insight into the possible involvement of these genes and their protein products in the growth control of malignant cells.

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Glycosylated phosphatidylinositols: their structures and roles in parasitic diseases

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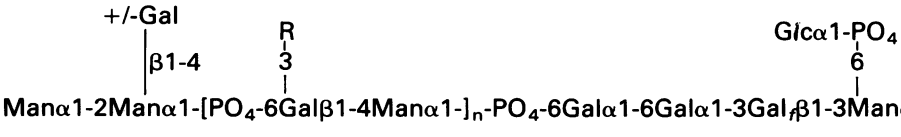
Glycosylphosphatidylinositol (GPI) membrane anchors appear to be ubiquitous throughout eukaryotic evolution, and are found attached to a wide variety of cell surface glycoproteins.[1–3] Structural studies on the GPI anchors of proteins from protozoa, yeast, and mammalian cells suggest that all anchors may contain the conserved core structure—ethanolaminephosphate-6Manα1-2Manα1-6Manα1-4GlcNα1-6-*myo*-inositol-1-PO_4-lipid—indicating the presence of a highly conserved biosynthetic pathway. In addition to these protein-GPI anchors, the parasitic protozoa (of the order Kinetoplastidia) synthesise a range of novel glycoconjugates which belong to the 'GPI family' through bearing the structural motif: Manα1-4GlcNα1-6*myo*-inositol-1-PO_4-lipid. These molecules include the lipopeptidophosphoglycans (LPPGs) of *Trypanosoma cruzi*[4] and the lipophosphoglycans (LPGs) and glycoinositolphospholipids of *Leishmania* parasites.[5–9] This latter group appear to represent specialised adaptations of the conserved GPI pathway and have no known counterparts in higher eukaryotes.

Work on the structure and function of these molecules has focused on the LPGs. The complete primary structures of LPG from *L major*, *L donovani*, and *L mexicana* have been determined by chemical and enzymatic modifications, one and two dimensional 500 MHz ^{1}H NMR, and fast atom bombardment mass spectrometry. The backbone structures of these molecules are highly conserved (figure). They all contain a variable number of phosphorylated oligosaccharide repeat units linked in linear array to a *lyso*alkyl-PI lipid moiety via a hexasaccharide core. The chains of repeat units are capped at the non-reducing terminus with a number of mannose-containing neutral oligosaccharides. Species-specific differences in these structures occur mainly in the nature and frequency of the side chains on the repeat units and in the composition of the cap structures.

Lipophosphoglycan appears to have an essential role in mediating the binding of parasites to epithelial cells in the midgut of the sandfly vector.[10] Binding to and detachment from these cells is dependent on the stage-specific expression of distinct oligosaccharide side chains on the LPG. We have also been interested in defining the role of LPG in the initial infection of macrophages in the mammalian host. Uptake of infective forms of the parasite by macrophages proceeds primarily via the complement receptors, CR1 or CR3, which bind to C3 components that opsonise surface LPG.[11] The efficient opsonisation of LPG with complement may depend, in part, on the presence of the terminal mannose cap oligosaccharides. We have shown that these caps, as well as intact LPG and live cells, are recognised by serum mannose binding protein (MBP-1), which is able to activate



Structure of lipophosphoglycans. R=H in L donovani; H or βGlc in L mexicana; H, [Galβ1–3]$_{0,3}$Galβ1-, or Arap1-2[Galβ1–3]Galβ1- in L major. The average number of repeat units (n) varies from 10 to 50.

complement via the classical pathway. Despite the presence of lytic complement components on the cell surface these infective forms of the parasite are resistant to complement mediated lysis. This seems to be due to an increase in the average chain length of the LPGs, which sterically prevents insertion of these components into the parasite membrane.

The second class of *Leishmania* specific GPIs are the glycoinositolphospholipids (GIPLs). These glycolipids are expressed in very high copy number on the surface of all developmental stages of the parasite. Three distinct families of GIPL have been characterised, which share structural homology with either the protein anchors (type 1), the LPG anchor (type 2), or both types of anchor (hybrid type). Although some of these glycolipids may act as precursors to the protein and LPG anchors, they appear to be predominantly metabolic end products.

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Poster abstracts

Serum IgA in IgA nephropathy has altered terminal oligosaccharide profile

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IgA nephropathy is a common renal disease characterised by the histological demonstration of mesangial IgA; the mechanism of this deposition is unknown. To investigate the possibility that altered IgA glycosylation contributes to its deposition we designed a lectin binding assay to assess the degree of terminal galactosylation of asparagine linked *N*-acetylglucosamine (GlcNAc) moieties found in the hinge region of IgA1 and IgA2. The assay uses lectins from *Triticum vulgaris* (glcNAc specific) and *Erythrina cristagalli* (D-galactose specific).

Anti-IgA coated microtitre plates were blocked with oxidised glutathione. Serum from 37 patients with IgA nephropathy and 34 age and sex matched controls was applied at a dilution that saturated the IgA binding capacity of the plates. Peroxidase conjugated lectins were then applied and the colour developed with *o*-phenylenediamine substrate. Results were expressed as the ratio of absorbances at 492 nm of *Triticum vulgaris* to *Erythrina cristagalli*.

Patient IgA gave a higher ratio than controls ($1{\cdot}53 \pm 0{\cdot}02$ *v* $1{\cdot}44 \pm 0{\cdot}02$, $p = 0{\cdot}007$). An IgA deficient serum gave background absorbances in both assays.

We conclude that serum IgA in IgA nephropathy has an abnormal glycosylation profile. This may have functional implications, varying the capacity to form complexes or combine with mesangial components, which might influence mesangial IgA deposition in IgA nephropathy.

Abnormalities in the oligosaccharide moieties of IgG in patients with myotonic dystrophy

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Myotonic dystrophy (MyD) is known as an autosomal dominant disorder transmitted by a pathological gene located on the long arm of chromosome 19 and is considered to be a 'membrane disease' affecting multiple organs. A decrease in serum IgG is one of several clinical manifestations of this disease. We found changes in the glycosylation profiles of IgG in 18 patients with MyD. The structural changes of oligosaccharide moieties were characterised by a marked increase in agalactosyl oligosaccharides and a decrease in digalactosyl oligosaccharides.

Similar glycosylation profiles have been reported in chronic inflammatory disease (CID). In CID, however, the total number of galactose residues attached to IgG molecules per millilitre of serum was the same as that in normal control IgG owing to the increased serum IgG concentration in CID. In contrast, in MyD the total number of galactose residues attached to IgG molecules per millitre of serum was markedly decreased, as decreased IgG concentration in serum is characteristic in MyD. Furthermore, the ratio of oligosaccharide F (having galactose on the Man α1–6 arm only) and oligosaccharide G (on the Man α1–3 arm only) was about 1·3 in patients with MyD, whereas it was 2·3 in CID and normal controls. These data suggest that the mechanism of incomplete galactosylation of IgG seen in MyD is different from that seen in CID.

Aglycosylated chimeric human IgG3 can trigger the human phagocyte respiratory burst

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We investigated the capacity of a complexed aglycosylated chimeric human IgG3 antibody to induce the respiratory burst in human monocyte-like U937 cells. We showed that the aglycosylated antibody, prepared by cell culture in tunicamycin, retained significant capacity to trigger this effector function which was assayed as superoxide generation.

Erythrocytes sensitised with near maximal levels of aglycosylated IgG3 were able to trigger >80% of the superoxide generation triggered by the glycosylated antibody from U937 cells induced to differentiate by interferon γ. The aglycosylated IgG3 gave half maximal responses at sensitisation levels only 72% higher than those required by the glycosylated form. Aglycosylated IgG3 was, however, much less effective in triggering superoxide generation by interferon γ treated U937 cells at low sensitisation levels as threshold responses required only 60 glycosylated IgG3 molecules per erythrocyte compared with 16 000 aglycosylated molecules.

The possible retention of significant biological activity by aglycosylated IgG may be an important consideration in the design of antibodies for use in diagnosis and treatment.

Expression of (sialyl)-Lewis X groups on human α acid glycoprotein during acute and chronic inflammation

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In acute and chronic inflammation human α_1 acid glycoprotein (AGP) is subject to marked changes in glycosylation. One of these changes is the degree of α1→3 fucosylation. This was detected by the affinity of AGP to *Aleuria aurantia* lectin (AAL) in crossed affinity immunoelectrophoresis. Five forms of AGP can be distinguished differing in their AAL reactivity (A0: non reactive with AAL, A1–A4: reactive with AAL). The forms A3 and A4 of AGP, especially, were transiently increased after laparotomy, severe burning, and primary section. A constitutive increased level of these two forms was detected in chronic inflammation (rheumatoid arthritis) relative to control serum.

The acute phase induced increase in fucosylation of AGP represented, at least partly, an increased expression of sialyl-Lewis X determinants on AGP. This was shown by staining with CSLEX-1. AGP was isolated by immunoaffinity chromatography and was fractionated in a non-reactive (A0) and four reactive fractions with AAL (A1–A4) by preparative crossed affinity immunoelectrophoresis.[1] Carbohydrate analyses of the AGP fractions showed that A1–A4 contained fucose in variable amounts, but A0 was not fucosylated at all. Staining of the AGP fractions on Western blots with CSLEX-1 showed that the expression of sialyl-Lewis X groups on AGP correlated directly with the degree of fucosylation of the AAL reactive fractions A1–A4.

Because the interaction between leucocytes and inflamed endothelium is mediated via sialylated Lewis X structures on leucocytes and the endothelial selectin ELAM-1 we postulate that the inflammation induced increase in the AAL reactive fractions of AGP, at least for acute inflammatory processes, represents a physiological feedback response.

1 Van Dijk W, Van der Stelt M E. In: Breborowicz J, *et al*, eds. *Affinity electrophoresis: principles and application*. Cleveland: CRC Press, 1991: 81.

A histochemical study of lectin binding in human developing and term placenta

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Successful fetal development in human pregnancy depends on efficient placentation, which enables the embryo to gain rapid access to the maternal circulation. This is achieved by the invasion of highly specialised trophoblast cells into the endometrium. Cell surface glycosylated molecules are known to be involved in cell-cell and cell-substrate interactions. This expression and distribution on trophoblast may contribute significantly to the invasive property.

We studied the binding of various biotinylated lectin probes—concanavalin A (Con A), *Sambucus nigra* (SNA), and *Ricinus communis* agglutinin (Ricin)—on frozen sections of first trimester and term placenta. The specificity of these lectin probes was determined by studies with the appropriate inhibitory sugar(s).

The results showed some difference in staining patterns between term and first trimester sections on the apical trophoblast membrane. This suggests an increase in glycosylation of membrane components as pregnancy progresses. With all three lectins there was a 100% inhibition of staining with the appropriate inhibitory sugar(s). These studies into the glycosylation patterns and saccharide expression give valuable insight into trophoblast invasion.

Analysis of the effects of IgG glycosylation status on polyclonal rheumatoid factor binding

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Alterations in the glycosylation state of the IgG molecule as found in patients with rheumatoid arthritis provide us with a possible mechanism for binding by rheumatoid factors. In rheumatoid patients the Fc oligosaccharides may be deficient in the normally terminating galactose, leading to changes in its antigenicity. This may operate through three mechanisms: (*a*) the missing sugars reveal a pocket that may allow other molecules with accessible sugars to bind, for example, Fab sugars on IgG; (*b*) the missing sugars disclose hidden sequences allowing rheumatoid factors to bind; (*c*) there is a conformational change to the Fc caused by the missing sugar.

IgG preparation: Is it important when determining glycosylation status?

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Previous studies have shown that Fc oligosaccharides of patients with rheumatoid arthritis are commonly missing galactose, thus exposing *N*-acetylglucosamine (GlcNac) at the terminus.

Normal and rheumatoid IgG have been isolated using salt fractionation followed by ion exchange chromatography. This purification method may mean that interesting subclasses of IgG are lost as an added negative charge due to sialylation could cause deviations in elution patterns. We used other means of purifying IgG using a protein G column, which binds all subclasses of human IgG and is not dependent on charge.

A comparison of dot blot analyses of normal and rheumatoid arthritis IgG prepared by ion exchange and protein G has been made. GlcNac was detected with *Bandeiraea simplicifolia* II and galactose by *Ricinus communis* agglutinin. Identical patterns of lectin binding were observed with both purification methods. The rheumatoid IgG, however, were found to be significantly different from the normal IgG (ion exchange: $p<0.01$, protein G: $p<0.037$).

When 10% sodium dodecyl sulphate/polyacrylamide gel electrophoresis separation and Western blotting were used a more detailed analysis of the IgG molecules for lectin binding was possible. Preliminary findings indicate that different patterns of IgG/lectin binding are present when IgG is purified by the two methods (ion exchange and affinity).

Investigation of the potential mechanisms controlling glycosylation changes in rheumatoid arthritis

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Oligosaccharides are an integral component of the immune system, and glycosylation changes may occur both normally or in association with disease. We have previously reported that serum IgG galactosylation and lymphocytic galactosyltransferase (GalTase) are reduced in rheumatoid arthritis (RA), and that a humoral regulatory component may be associated with these changes. To investigate the potential mechanisms controlling these glycosylation changes we studied the interrelation between (*a*) lymphocytic GalTase and agalactosylated IgG (Gal(0)) and (*b*) lymphocytic GalTase and IgG anti-GalTase antibodies.

The interrelation between lymphocytic GalTase and Gal(0) was positive and linear ($p<0.05$) in 18 healthy subjects and 14 with non-autoimmune arthritis, and negative and linear ($p<0.05$) in the group with RA. The difference between these two correlations was significant ($p<0.01$), suggesting a positive feedback homeostatic mechanism regulating Gal(0) level in normal subjects, which is disrupted in those with RA.

A longitudinal study of the interrelation between lymphocytic GalTase and IgG anti-GalTase antibodies in 13 patients with RA showed a significant association between increased IgG anti-GTase antibody levels and reduced lymphocytic GalTase activity (B cell $p<0.05$ and T cell $p<0.01$). These data suggest that IgG anti-GalTase antibodies are functionally related with the down regulation of GalTase and may be part of an aberrant glycosylation network associated with the pathogenesis of RA.

These data indicate the presence of a network of regulatory elements involved in IgG galactosylation, which are of relevance to the pathogenesis of RA, and may be an integral component of a glycosylation mechanism controlling the carbohydrate content of other immunologically pertinent molecules.

Variation in serum and lymphocytic glycosyltransferase activity in rheumatic disease

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Control of protein glycosylation is thought to be abnormal in rheumatoid arthritis (RA). In particular, serum agalactosyl IgG is increased, whereas there is a decrease in lymphocytic galactosyltransferase (GalTase) activity.[1] To determine whether there are other glycosylation changes associated with arthritis in general, both serum sialyltransferase (STase) and GalTase were determined in 10 patients with RA, nine with ankylosing spondylitis (AS) 10 with systemic lupus erythematosus (SLE), and 10 with osteoarthritis (OA), and the results compared with those for 20 healthy subjects. Lymphocytic STase activity was also measured in 10 patients with active RA and compared with that in 10 healthy controls.

Serum STase activity was raised in all the diseases tested (RA 34·6%, $p<0.001$; AS 30·3%, $p<0.001$; SLE 21·3%, $p<0.01$; OA 18·1%, $p<0.01$); in contrast, serum GalTase activity was only increased in AS (42·0%, $p<0.001$) and OA (17·8%, $p<0.05$). Drug treatment did not influence glycosyltransferase activity, and no correlation was found between STase and GalTase activity, nor was there an association of glycosyltransferase activity with age. In contrast, there was a significant decrease (−41%) in STase activity (6.97×10^3 (1·63) cpm/mg protein, mean (SEM)), in RA lymphocytes when compared with controls (11.82×10^3 (1·23) cpm/mg protein, $p<0.05$). Drug treatment and sex of the patient did not affect enzyme activity.

These data indicate that glycosylation changes occur in association with a variety of arthritic diseases and that they are not restricted to lymphocytes. Furthermore, the data suggest differential disruption in glycosylation control in the rheumatic diseases as there are wide variations in serum glycosyltransferase activities. In particular, an increase in GalTase was not found in RA or SLE. This suggests that there may be distinct protein glycosylation patterns associated with different arthritic diseases.

1 Axford J S, Sumar N, Alazi A, Isenberg D A, Young A, Bodman C B, *et al.* Changes in normal glycosylation mechanisms in autoimmune rheumatic disease. *J Clin Invest* 1992; **89**: 1021–31.

Characterisation of extracellular sialytransferase activity in human B lymphocytes

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The existence and role of extracellular sialyltransferase activity has been discussed controversially. In lymphocytes *ecto*-α-2,6-sialyltransferase (α-2,6-STase) activity has been proposed to modulate glycosylation of cell surface antigens. By means of an antiserum against α-2,6-STase we showed that in B lymphocytes α-2,6-STase is confined to the Golgi compartment. We found, however, that

established B cell lines constitutively secrete STase activity in various amounts. We investigated several B cell lines derived from different maturation stages (NALM-6, pre-B cell; JOK-1, lymphoblastoid cell; U266, plasma cell). STase activity was measured on intact cells and cell lysates with CMP-[^{3}H]-NANA as substrate. STase activity of NALM-6 cells was marginal, whereas in JOK-1 and U266 increasing STase activity was found. When an external acceptor (asialofetuin) was used STase activity was also identified in the culture supernatant. This STase activity specifically sialylated N-linked oligosaccharide chains applying α_1 acid glycoprotein as acceptor. Cell surface sialylation by the external STase was assessed by using fluoresceinated CMP-NANA and monitoring the increase of cell surface label in a FACSCAN cytofluorometer. Incorporation of fluoresceinated NANA into cell surface structures could be enhanced by prior treatment of the cells with neuraminidase. Although the biological significance of this extracellular STase activity remains unclear at the moment, it is remarkable that secretion of STase increases during B cell maturation.

Generation of a group of sialylated B lymphocyte differentiation antigens by human Golgi β-galactoside α-2,6-sialyltransferase

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The role of the human β-galactoside α-2,6-sialyltransferase (α-2,6-STase) in the generation of B cell surface antigens was investigated by selecting subclones of COS cells constitutively expressing a transferred cDNA which encodes the human α-2,6-STase. Expression of human α-2,6-STase in COS cells was sufficient to generate sialylated cell surface epitopes on different glycosylated antigens recognised by monoclonal antibodies to CDw75, CD76, and the unclustered monoclonal antibodies HB-4 and EBU-65. These epitopes were sensitive to neuraminidase treatment and are likely to contain terminal α-2,6-linked sialic acid residues. A new antiserum raised against bacterially expressed human α-2,6-STase fusion protein was used to localise the sialyltransferase in two cell lines with high expression of either endogenous (B cell line JOK-1) or recombinant (COS α-2,6-STase cells) human α-2,6-STase. In both cell lines the enzyme was detected only intracellularly in the juxtanuclear region and not on the cell surface. In contrast, CDw75, formerly proposed to be identical with an *ecto*-α-2,6-STase, was strongly expressed on the cell surface. The B cell line IM-9, negative for human α-2,6-STase mRNA was also negative for this group of antigens. The different expression patterns show that neither the CDw75 nor any of the other sialylated antigens analysed are identical with the human α-2,6-STase. The presence of a surface-expressed α-2,6-STase seems unlikely in these cells. Sialylation of these antigens is generated in the Golgi compartment. We propose that CDw75, CD76, HB-4, and EBU-65 represent a group of B cell differentiation antigens which require the activity of α-2,6-STase in COS and B cells.

Structure and possible function of proteoglycans of human B lymphocytes

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Human B lymphocytes synthesise predominantly proteoglycans with chondroitin sulphate chains (CSPG). The B lymphoblastoid cell line LICR-LON-HMy2 produces two CSPG, isolated from cell lysates and culture supernatant, which differ in the primary structure of their protein cores. Both proteoglycans consist of a protein core of 30 kilodaltons to which four chondroitin sulphate chains each of 26 kilodaltons are attached. For both CSPG a molecular mass of about 135 kilodaltons was deduced from the hydrodynamic sizes of the glycosaminoglycan chains obtained after β elimination and migration of the protein cores in sodium dodecyl sulphate/polyacrylamide gel electrophoresis. Differences in proteoglycan synthesis during B cell differentiation were assessed by analysing B cell lines which represent different maturation phases, from the pre-B to the plasma cell stage. We found that all cell lines investigated synthesise CSPG with a protein core of approximately 30 kilodaltons. Differences were observed both in size of glycosaminoglycan chains, composition, and sulphation of sugars. The size of glycosaminoglycan chain and sulphation of chondroitin sulphate increased concomitantly with maturation. Only for the cell line U266 (equivalent to the plasma cell stage) did we find a significant amount of proteoheparan-sulphate (40% of proteoglycans in the cellular compartment).

CSPG of B cells may be involved in adhesion of lymphocytes to extracellular matrix (ECM) since they bind to matrix components, e.g. collagen and fibronectin. Cell surface expression of CSPG, as assessed by binding of monoclonal antibodies against CSPG, parallels the ability of B cells to adhere to ECM. The ability to adhere to ECM gradually declines during differentiation. U266 cells do not express significant amounts of CSPG and at the same time do not adhere to ECM. Secreted CSPG competitively inhibit binding of cell bound CSPG to ECM constituents.

We tentatively conclude that B cell CSPG have a role in stage dependent adhesion to ECM. The adhesion may be regulated by modulation of the carbohydrate structure and by enhanced secretion of CSPG.

Modulation of macrosialin, a macrophage restricted late endosomal glycoprotein, by endocytosis

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Macrosialin, a new macrophage (MØ) restricted glycoprotein identified through its reactivity with monoclonal antibody FA.11,[1] is primarily localised to the late endosomal compartment of these cells (Rabinowitz S S *et al*, unpublished data). The antigen (Mr 87–104 kilodaltons) is composed of a core peptide of 44 kilodaltons and, more importantly, the sugar moiety can be differentially modulated according to the MØ functional state (Rabinowitz S S, Gordon S, unpublished data). In exudate MØ, macrosialin is the main wheat germ agglutinin binding protein, and the alternative glycosylation undergone by the molecule accounts for differential binding of the lectin by these cells (Rabinowitz S S, Gordon S, unpublished data).[2]

We recently cloned and sequenced the protein and characterised it as a new member of the glycoproteins (lgp or lamp) family. The open reading frame codes for a protein 326 amino acids long, composed of a signal peptide, two intralumenal domains separated by a proline-rich hinge region, a transmembrane span, and a cytoplasmic tail. As with the other lamps it is the domains which are closest to the membrane that are the most homologous to the other members of this family of highly conserved glycoproteins. In addition, macrosialin was shown to be the murine homologue of the human macrophage protein CD68.

We also investigated macrosialin expression and glycosylation in cell culture. Phagocytic stimuli, such as zymosan and BCG, enhance expression of total antigen as measured by ^{125}I monoclonal antibody binding. Adhesion to non-specific substratum, uptake of latex beads, and treatment by mannan (10 mg/ml) induce only low levels of macrosialin. Pulse-chase experiments using [^{35}S]methionine and [^{3}H]mannose show that zymosan and BCG do not enhance synthesis of FA.11, but that the immune precipitated molecule is threefold more heavily glycosylated than that in latex ingestion and other controls. The new glycoform has a substantially prolonged half life in phagocytosing MØ, suggesting that alternative glycosylation of macrosialin controls its rate of degradation and resultant levels of expression in MØ.

Differential processing of macrosialin seems to be specific as F4/80—a macrophage restricted plasma membrane glycoprotein, and lamp-2—a widely distributed lysosomal marker also heavily glycosylated, fail to show any significant increase of specific label. Because of its cellular restriction, compartmental localisation, and molecular versatility, macrosialin is a natural candidate in mediating normal MØ endocytic function, which could be conveniently exploited by pathogens in subverting MØ killing mechanisms.

1 Smith M J, Koch G L E. Differential expression of murine macrophage surface glycoprotein antigens in intracellular membranes. *J Cell Sci* 1987; **87**: 113–9.

2 Rabinowitz S S, Gordon S. Differential expression of membrane sialoglycoproteins in exudate and resident mouse peritoneal macrophages. *J Cell Sci* 1989; **93**: 623–30.