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The importance of oligosaccharides to rheumatic disease: a personal perspective

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In 1991 I wrote an editorial for this journal [1] detailing my reasons for thinking that changes in the glycosylation of proteins were relevant to disease mechanisms in rheumatology. The potential biological importance of oligosaccharides and the considerable degree of heterogeneity that these highly variable groups of branched ring structures confer to the protein backbone was discussed. The main focus of the editorial concentrated upon how oligosaccharides could be relevant to both normal and disease associated biological mechanisms and how interference with the oligosaccharide/protein interaction may have therapeutic implications.

The last 4 years have seen an explosion of activity in these two areas. On the functionality front, there is frantic interest by pharmaceutical companies to understand, and thus manipulate, the mechanisms of protein glycosylation in order to alter the pharmacokinetic and dynamic profiles of their products. Therapeutically, intensive research has been focused on the development of carbohydrate compounds, such as the simple oligosaccharide structure known as sialyl-Lewis^x, which has been shown to have dramatic protective effects in a P-selectin dependent model of lung injury [2]. This area of research is particularly exciting, since these synthetic compounds and their analogues have enormous potential and can be targeted at a wide variety of therapeutic applications.

This interest is also reflected by the appearance in the bookstalls of two new journals dedicated to glycosylation research (Glycobiology and The Glycosylation and Disease Section of the *Glycoconjugate Journal*), together with a deluge of glycobiology meetings; including two dedicated to glycoimmunology [3, 4]. Furthermore, the research division of the European Union have recognized the need to understand the relationship between glycosylation and arthritis and are currently funding a European community concerted action, named EUROCARB, for this purpose. Eurocarb was also a major sponsor of the 1st Electronic Glycocosci Conference held in 1995.

So what is the current state of play?

Carbohydrate measurement

Improved technology has provided researchers with two automated carbohydrate analysers. One is based on hydrazinolysis and subsequent fractionation of radio labelled and desialylated oligosaccharides (Oxford Glycosystems) and the other is based on high pH anion exchange, coupled with electrochemical detection (Dionex Carbohydrate System), which allows determination of the monosaccharide composition as well as mapping of the oligosaccharide moieties.

If you require a much more detailed structural analysis, fast atom bombardment and electrospray mass spectrometry can be powerful tools for defining structural features in glycoproteins, such as site-specific glycosylation [3]. Once the basic structure of the oligosaccharide under investigation is known, then simpler techniques can be devised which enable comparative analysis to take place. These analyses are of potential importance as they help shed new light on the pathogenesis of diseases which may have disparate phenotypes, but which occur as a result of similar pathological mechanisms.

To date, most interest in the rheumatological field has focused on the N-linked complex sugars associated with the IgG molecule and two techniques are commonly used to determine the relative abundance of the terminal sugars.

Firstly, monoclonal antibody (MoAb) detection of *N*-acetylglucosamine (GlcNAc) can be employed to reflect the degree of IgG agalactosylation by extrapolation from comparative sequence data [5].

Secondly, analysis using lectins, which are specific for different sugar residues *eg*:

- *Bandeiraea simplicifolia II* for *N*-acetylglucosamine residues [6]
- *Psathyrella velutina* for *N*-acetylglucosamine β1-2mannose residues [7]
- *Ricinus communis agglutinin* for galactose residues [6]

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- *Aleuria aurantia* for fucosylated lactosamine units [8]
- *Concanavalin A* for biantenary or high mannose N-linked glycans [9]
- *Sambucus nigra* and *Maackia amurensis* for sialic acid [3]

can be utilized to determine their relative abundance and linkage.

Another technique which is currently being investigated in relation to this field, is fluorophore assisted carbohydrate electrophoresis, which can be used to obtain an overall oligosaccharide profile of the glycoprotein of interest. This method utilizes the quantitative fluorophore labelling of reducing saccharides, removed from the protein backbone, which are then separated by high resolution polyacrylamide gel electrophoresis [3].

A number of uncertainties however creep in as the analytical techniques employed become simpler, even when the basic oligosaccharide structure is known.

How certain are you about what you are measuring?

It is important to be aware when analysing or interpreting data generated by these simpler techniques, that there are a number of possible drawbacks and potential inaccuracies associated with each of the following steps in the analytical procedures.

Purification of immunoglobulin

The type of oligosaccharide (*ie* high mannose or complex) and the site of attachment (*ie* heavy or light chain) can, for example, influence: the overall tertiary protein structure, the Fc effector function, and the antigen binding affinity of IgG [10–13]. This, together with the fact that the overall charge density of IgG will vary depending upon the degree of sialylation, means that the choice of purification method for IgG is highly likely to influence the population of IgG molecules obtained.

Indeed, comparison of two conventional purification techniques, protein G affinity and DEAE ion exchange chromatography [14], has demonstrated that differences in the populations of IgG molecules obtained can occur. Comparison with isolation methods based on solid phase capture of IgG from whole serum using protein A/G [5] or anti-IgG antibody [15], has not yet been carried out. It is important that this is done to ensure that similar populations of IgG are being studied.

Unfortunately, this is only where the uncertainties begin, for in diseases such as rheumatoid arthritis (RA), it is notoriously difficult to be certain as to exactly what you have purified or captured for analysis. Undoubtedly, a certain amount of complexed material will have been isolated along with the IgG, such as IgM, IgA and complement components.

These possible contaminants, despite probably being present at very low concentrations, are often highly glycosylated, and it is therefore imperative to ensure that the glycosylation changes detected are IgG derived and not from contaminants. All studies should therefore incorporate additional analytical analysis *eg* Western blot, in parallel with the carbohydrate investigation, to make sure that major carbohydrate shifts do not occur concomitantly with idiosyncratic protein changes.

Carbohydrate detection

Glycosylation changes may be tissue specific [16, 17], so it is important to ensure that the appropriate clinical samples are being investigated. Also, it is probably unwise to assume that the pattern of disease associated glycosylation changes (*ie* number, type, composition and distribution) are analogous to those previously reported in different disease and in certain racial groups. Our investigations indicate that this concern is probably well founded [18] and emphasizes the point that all investigations should be carried out on the assumption that the glycosylation changes that occur may be unique to the tissue or disease under investigation, unless detailed analysis prove otherwise.

To avoid confusion it would also seem sensible to report the raw data generated by a particular analytical method, rather than entangle data in formulae, generated using other methods or other disease groups, so that inferences can be made about the presence/absence of certain oligosaccharides [19]. If inferences are required then it is imperative to ensure that what is being measured by one technique is also being detected by another. There are potentially a number of drawbacks when using MoAbs or lectins, for example, MoAb may have differential affinity for biantennary oligosaccharide structures dependent upon the terminal sugar, by reasons of accessibility, and lectins may not always only bind to the terminal oligosaccharide residues [15, 19].

It is my view that in order to eradicate the danger of misinterpretation and loss of vital data, a panel of lectins, or monoclonal antibodies if they are available, with various sugar and linkage specificities should be used, to determine the abundance of each monosaccharide individually. Indeed a comprehensive analysis of this type is essential when investigating diseases for the first time or when comparing different diseases, as in the case of a report by Hernandes Pando *et al.* [20]. Here, the authors present data on Mexican patients with Takayasu's arteritis in which serum IgG glycosylation, as measured by a monoclonal antibody against GlcNAc is shown to be similar to patients with Mycobacterial tuberculosis (MTB) infection. This is achieved by capturing IgG from serum using protein A. Then converting the degree of monoclonal antibody binding to represent % agalactosyl IgG, by correlating the absorbance with the number of biochemically released divalent oligosaccharides that lack terminal galactose residues, that have previously been derived from the IgG of

patients with RA and MTB. They suggest that Takayasu's arteritis is probably caused by a Mycobacterial infection as these patients also have a humoral immune response to a 38 kDa MTB protein, and to the 65 kDa heat shock protein of *Mycobacterium-lepri*. They may well be right, but what is certain is that these data will certainly form the basis for lively discussion.

To help resolve some of the above drawbacks, EUROCARB is co-ordinating firstly a comparative study of IgG glycosylation techniques, to determine whether what has been reported by one laboratory can also be measured by another, and secondly the production of IgG standards to be distributed to interested laboratories.

There is continued interest in immunoglobulin G

The interest within the rheumatology community continues to be focused on IgG and this is probably due to more than habit. For the wealth of data implicating glycosylation as a modulator of IgG function [21], rheumatoid factor binding [22, 23] and immune complex formation [24] is compelling to say the least. But if you are not an immunoglobulin fan, then you do not need to be reminded of the fact that, there is a whole array of other immunologically pertinent members of the immunoglobulin super family *eg* the MHC [25], T cell receptor [26] and CD22 [27], which are potential fruitful fields of glycosylation research.

What about other molecules?

A whole new area of research has been opened up by the recent publication of data on the following molecules.

α1-acid glycoprotein [8]

This acute phase reactant changes its glycosylation profile during inflammation, as manifested by an increase in sialyl-Lewis^x substitution. This is thought to be an anti-inflammatory physiological feedback response to the selectin mediated interaction between leukocytes and the inflamed endothelium.

Haptoglobin

Haptoglobin is secreted by the liver in a variety of inflammatory conditions and interestingly, undergoes disease specific glycosylation changes (G. Turner, personal communication). In RA there is an increase in the fucose content but no change in galactose [28], whereas in Crohn's disease, there is an increase in the sialic acid content and in bronchopneumonia, the glycosylation of Haptoglobin is unaltered and is similar to those found in healthy individuals. The data tantalizingly imply that these subtle changes could be related to the functional role played by this glycoprotein.

Ferritin [9]

Different glycoforms of plasma Ferritin have been identified in Adult Still's Disease (ASD). The glycoforms are basic with a reduced mannose content in active disease, compared to inactive ASD and other systemic diseases. It is thought that the presence of these glycoforms may offer an additional diagnostic tool for Still's Disease, and hopefully, more common conditions.

Do alterations in carbohydrates affect glycoprotein function in the same way?

The simple answer to this is no [29]. Intriguingly, the evidence indicates that similar glycosylation changes on different protein backbones can give rise to very different modulatory effects. For example, decreased glycosylation can cause a graded increase in receptor binding and activity of granulocyte/macrophage colony stimulating factor and increased activity of Interleukin-4. In contrast, the absence of CD4 glycosylation results in endoplasmic reticulum retention and degradation and similarly glycosylation absence on MHC Class II molecules results in variable loss of antigen presenting functions. The only common feature of the varied functions of oligosaccharides is that they either mediate specific recognition events or they result in modulation of biological processes.

So what does the future hold?

Significant effort is being made to capitalize upon the potential therapeutic effects that may be derived from synthetic oligosaccharide structures and modulation of glycosyltransferase activities.

I have already discussed how synthetic sialyl-Lewis^x may be used, but let us consider the glycosyltransferases briefly. It is known in RA that there is a breakdown in the homeostatic events regulating lymphocytic galactosyltransferase activity [30], and it is thought that the mechanisms regulating galactosylation of proteins, is simply not an all or nothing event occurring systematically throughout the whole body, but is perhaps a finely tuned process that is tissue specific [16]. This is well illustrated in the case of the fucosyltransferase family of enzymes, that are essential for cell adhesion and where there are at least seven genes encoding the enzyme that have differential tissue expression [17].

Interleukin-6 is a cytokine that has been shown to be involved in the regulation of the glycosylation of acute phase proteins. Differences have been found between RA and SLE patients in studies investigating this regulatory mechanism, and cytokine modulation is going to be, not only an interesting area of research as a whole, but important when more is understood about how it is associated with protein oligosaccharide changes [31].

Back to immunoglobulins, glycosylation changes in the second complementarity region has been shown to affect antibody affinity for antigen in a manner suggestive, that not only the site of glycosylation, but the type of oligosaccharide (*ie* high mannose or N-linked complex) may be critical [13]. Furthermore, recent evidence suggests that post-translational glycosylation modification of immunoglobulin, having the same amino acid sequence, may cause them to change from being poly- to mono-specific [32].

Charting how sugar profiles fluctuate as part of normal physiological mechanisms will be crucial if we are to understand what is happening in disease. Studies on relatives of those with disease [33] or individuals pre-destined to develop autoimmune disease, such as the Pima and Papago Indians [34] will be significant in furthering our understanding of these diseases.

Markers of RA and predictors of disease outcome are crucial aids to the provision of optimal treatment for individual patients. IgG galactosylation has been shown to be a useful early marker of RA [35] and its level of reduction appears to be an indication of poor outcome [36]. These reports indicate that instituting a rapid glycosylation assay in the protocol for the investigation of early synovitis may prove very useful.

The intention of this editorial has been to give further insight into current developments in glycoimmunology. If I have whetted your research appetite, then I look forward to discussing this exciting field with you personally [4] and the next Glycoimmunology meeting http://www.sghms.ac.uk/conference/glyco/intro.htm.

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