LYMPHOCYTIC GALACTOSYLTRANSFERASE IN RHEUMATOID ARTHRITIS AND OTHER DISEASES

A THESIS SUBMITTED BY

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"ALWAYS EXPECT THE UNEXPECTED"

Dr Robert S Schwartz.
Boston, July 1989.
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ABSTRACT.

IgG-Fc galactose (IgG-gal) levels varies with age and reduced serum IgG-gal values have been reported in a restricted number of diseases: rheumatoid arthritis (RA), tuberculosis (TB), Crohn’s disease and SLE associated with Sjögren’s syndrome.

The experimental objective of this study was to determine whether lymphocytic galactosyltransferase (GTase), the enzyme responsible for the transfer of galactose to IgG-Fc within the lymphocytic golgi, is defective in RA, and other diseases, and if it is causally related to IgG carbohydrate abnormalities.

A reliable and reproducible assay of lymphocytic GTase, detecting the transfer of radiolabelled galactose to an acceptor glycoprotein, was devised, with a coefficient of variability of 4.4% and the ability to detect GTase from about 5x10^5 cells.

Preliminary experiments with human GTase showed that activity could be detected in RA and control lymphocytes, serum and plasma, although no correlation existed between serum and lymphocytic levels in RA.

A control age-matched population (n=32), without autoimmune disease, was used throughout for comparison. In all, except three, IgG-gal values were within 2 standard deviations of the normal mean for the age. No age relationship between IgG-gal and GTase activity was found in the control population. A wide range of GTase activities was noted in the control population and IgG-Fc galactosylation was positively correlated with GTase activity. A similar level of GTase activity was observed in B cell enriched preparations from human tonsils.

The GTase activity in whole mononuclear cell preparations, from patients with RA, was significantly elevated. However, removal of monocytes, resulted in a fall in GTase activity, and B and T GTase activities were both reduced when measured individually when compared to healthy controls. This may indicate that T cell glycoproteins could be affected in a similar manner as IgG. A positive correlation between B and T cell GTase activity and a negative correlation between IgG-gal and B and T cell GTase was found.

On splitting the rheumatoid arthritis population into groups, dependent upon drug treatment, it was noted that those patients taking sulphasalazine (SASP) had higher mean levels of GTase than the other patients in the study. This effect was shown not to be due to disease activity and was therefore presumed
to be related to the actions of SASP itself. SASP is known to have pharmacological potential to cause these effects.

In an initial longitudinal analysis of glycosylation parameters in patients with rheumatoid arthritis, a temporal relationship with clinical activity was found. In addition, SASP was shown to maintain GTase levels in other patients. Abnormal GTase activity was observed in clinically inactive patients, which may indicate continuing subclinical cellular dysfunction. Although GTase activity was found to be normal in patients with osteoarthritis when compared to healthy controls, it was decreased in patients with SLE, Crohn's disease and active pulmonary TB. In only the TB patients did GTase return to normal with treatment.

CD5 positive B cell GTase activity from patients with chronic lymphocytic leukaemia was determined and a significant decrease in GTase activity was noted when compared to a control population with the same age range.

In an attempt to investigate the mechanism of fluctuations in GTase activity the presence of an intracellular inhibitor and specific polymorphisms within the gene encoding the GTase activator (GTA) gene, were looked for.

Using normal GTase from human lymphocytes, bovine milk and human milk, no inhibition of activity was detected when mixed with RA lymphocyte extract. In one experiment, synergism was noted in both RA and normal preparations, which may indicate the presence of a co-factor in human lymphocytes.

On restriction enzyme digestion of RA and normal lymphocytic DNA, no gross structural changes in the GTA gene was seen and hence gene abnormalities are also unlikely to be responsible for reduced GTase activity. RFLPs were, however, observed using the enzyme Bgl II in both normal and RA DNA.

To test whether fluctuations in isoenzymes of GTase may be implicated in the abnormal glycosylation of IgG, and potentially other glycoproteins, GTase activity towards 5 different oligosaccharide acceptors was determined. A decrease in enzyme activity, comparable to ovalbumin, was noted only with asialo-mucin.

In order that cells, other than those from the periphery, could be studied in disease, the MRL/lpr mouse, a model for rheumatoid arthritis, was used. GTase from pooled peripheral and splenic B cells was assayed from MRL/lpr, MRL+/+ and CBA/J (control) strains of mice. Splenic B cell GTase activity was found to be similar in both the MRL strains and the CBA/J control strain, whereas enzyme activity was reduced in the peripheral B cells only in the MRL strains. This may suggest that reduced GTase in the MRL mouse is one of a
number of factors leading to disease in the lpr strain, which are not present in
the +/+ strain, and is not a direct result of the disease itself.
In conclusion, GTase activity has been studied in normal individuals and those
with RA, SLE, TB, CD and CLL. Reduced levels of GTase are apparent in the
presence of disease, irrespective of clinical activity, but in individuals with TB
the GTase always returns to normal following antibiotic treatment and often
does in patients with RA treated with SASP. There is no evidence to suggest
that reduced GTase activity may be due to an intracellular inhibitor of GTase in
RA, nor to gross structural changes in the GTA gene. Evidence is provided
which suggests that GTase isoenzyme changes may occur in RA. A hypothesis
is presented linking isoenzyme changes with glycoprotein abnormalities and the
clinical presentation of disease. Similar GTase activity findings are reported in
the peripheral, but not the splenic, B cells of the MRL (arthritic) mouse,
suggesting that cells being sampled in the periphery are trafficking from areas of
inflammation.
EXPERIMENTAL OBJECTIVES.

Rheumatoid arthritis (RA) is an autoimmune disease of unknown aetiology, in which the occurrence of rheumatoid factor and immune complexes are characteristic and may be causally related to the production of articular inflammation.

Although certain germline immunoglobulin genes may be preferentially utilised in rheumatoid arthritis, as inferred by the occurrence of public Vh gene idiotypes, no unique amino acid sequence has been detected in RA immunoglobulin, which may confer potential antigenicity.

It is known, however, that carbohydrate differences (galactose reduction) occur at the Fc region of IgG in RA, and that the differences can also be caused by Mycobacterium tuberculosis infection.

The experimental objective of this study was to determine whether the galactosyltransferase enzyme, which is responsible for galactose transfer to the IgG-Fc oligosaccharide in the endoplasmic reticulum of the lymphocytic golgi, was in any way defective in rheumatoid arthritis and hence possibly related to the pathogenesis of the disease.
CHAPTER 1.

INTRODUCTION.

1. Glycoproteins.

A. Overview.
Glycoproteins are distinguished by oligosaccharide chains covalently attached to a peptide backbone, and this may account for some of their characteristic physical and chemical properties. Glycoproteins are found in viruses, bacteria, fungi, green plants and in eukaryotic cells where they serve a variety of functions (1,2). Connective tissue glycoproteins, such as the collagens and proteoglycans are structural elements in various animal species, as are the cell wall glycoproteins of yeasts and green plants. The body fluids of vertebrates are rich in glycoproteins secreted from various glands and organs. The submaxillary mucins and the glycoproteins in the mucous secretions of the gastrointestinal tract, which consist of numerous oligosaccharide chains attached at closely spaced intervals to a peptide backbone, serve as lubricants and protective agents. Glycoprotein constituents of blood plasma include the immunoglobulins, the transport proteins transferrin, ceruloplasmin, and transcobalamin, as well as all the known clotting factors, and many complement components. Follicle-stimulating hormone, luteinizing hormone, thyroid-stimulating hormone and chorionic gonadotrophin are also all glycoproteins as are the enzymes ribo-nuclease, deoxyribonuclease, and α-amylase. Glycoproteins also occur as integral components of cell membranes in various species. Enveloped viruses contain surface glycoproteins that are involved in the attachment of virus to host, and in eukaryotic cells the histocompatibility antigens are membrane glycoproteins. There is increasing evidence that cell surface glycoproteins are involved in a number of physiologically important functions such as cell-cell interaction, adhesion and migration.(75-77,84).

B. The classification of glycoproteins.
Glycoproteins come in a variety of shapes and forms, and may be classified according to biochemical composition and linkage. This may not, however, relate to their function.
i. O-linked glycoproteins.
In many glycoproteins, the oligosaccharide chains can be attached to the peptide by an O-glycosidic linkage.

![Chemical structure of O-linked glycosidic bond](image)

**Figure 1a**
N-Acetylglucosamine linked to a serine residue by an O-glycosidic bond.

a. N-acetylgalactosamine-hydroxyl linkage to serine and threonine.
The submaxillary mucins, and fetuin, are common examples of this form of linkage.

1. Submaxillary mucins.
The following general structure is found:

\[
\begin{align*}
\text{NAN} & \xrightarrow{\alpha2,6} \text{GalNAc} & \xrightarrow{} \text{Ser(Thr)}
\end{align*}
\]

Where N-acetylgalactosamine (GAL NAc) is substituted at C6 by sialic acid (N-acetylneuraminic acid, NAN).
GalNAc ► Gal ► GalNAc ► Ser(Thr)

FUC

Neu5Ac

GlcNAc ► Gal ► GalNAc ► Ser(Thr)

FUC

Figure 1b.
Examples of submaxillary mucin from the pig (above) and the dog.
GalNAc = N-Acetylgalactosamine, GlcNAc = N-Acetylglucosamine, Gal = Galactose, FUC = Fucose, Neu5Ac = Sialic acid/neuraminic acid, Ser = Serine, Thr = Threonine.

Depending upon origin, the submaxillary mucin structure will vary, for example Figure 1b. No matter how complex these oligosaccharides may become (i.e. blood group substances) the core disaccharide never varies and sialic acid is always linked α2,6 to N-acetylgalactosamine.

2. Fetuin.
The following general structure is found:

\[
\text{Gal}^{\beta1,3} \rightarrow \text{GalNAc} \rightarrow \text{Ser(Thr)}
\]

\[\alpha2,3\] \[\alpha2,6\]

NAN

NAN

Where a second sialic acid (NAN) residue is attached to C6 of N-acetylgalactosamine (GalNAc).

b. Other forms of hydroxyl linkage to serine and threonine.
Oligosaccharides linked O-glycosidically from mannose to a serine or threonine residue in the peptide occur most commonly in glycoproteins from yeasts and fungi.
For example, the pentasaccharide found in the envelope glycoprotein of Cryptococcus laurentii (3).

\[
\begin{array}{c}
\alpha_{1,2} \quad \alpha_{1,6} \quad \alpha_{1,3} \\
\text{Man} \rightarrow \text{Man} \rightarrow \text{Man} \rightarrow \text{Man} \rightarrow \text{Ser/Thr} \\
\uparrow \beta_{1,2} \\
\text{Xyl}
\end{array}
\]

In the mycodextranase secreted by Penicillium melinii, which contains 25 carbohydrate side chains attached to serine or threonine, glucose may also occur in mannose-linked oligosaccharides (4).

\[
\begin{array}{c}
\alpha_{1,2} \quad \alpha_{1,2} \\
\text{Man} \rightarrow \text{Glc} \rightarrow \text{Man}
\end{array}
\]

Xylose is the connecting sugar on O-glycosidic linkage to serine or threonine in the linkage region of the proteoglycans. Attachment of oligosaccharide to serine and threonine can therefore involve a pentose, a methylpentose, or a hexose as well as the amino sugar N-acetylgalactosamine.

c. Hydroxyl linkage to hydroxylysine and hydroxyproline.
Collagens and basement membranes contain yet another type of glycoprotein linkage structure consisting of galactose and glucosyl-galactose disaccharides linked O-glycosidically to the hydroxyl group of hydroxylysine (Hyl) in the peptide (5), as follows:

\[
\begin{array}{c}
\beta_{1,2} \\
\text{Glc} \rightarrow \text{Gal} \rightarrow \text{Hyl}
\end{array}
\]

This disaccharide unit is found in a number of collagens, and the glycosylated hydroxylysine residues in vertebrate collagens always occur within the amino acid sequence Gly-X-Hyl-Y-Arg (x and y = amino acids) (6). The disaccharide containing glycopeptide from human glomerular basement membrane bears this configuration and carries the major antigenic determinants of humoral cellular anti-basement membrane autoimmunisation (7).
ii. N-linked glycoproteins.

Figure 1c
N-Acetylglucosamine linked to an asparagine residue by an N-glycosidic bond.

Glycoproteins containing oligosaccharide chains linked N-glycosidically from N-acetylglucosamine to the amide nitrogen of asparagine (Asn), are of two general types: simple, which contain only mannose (Man) and N-acetylglucosamine (GlcNAc) residues, and complex, which, in addition to those sugars, also contain sialic acid, galactose and fucose. A single glycoprotein may contain both simple and complex oligosaccharide chains, as do thyroglobulin (8) and IgM (Dr T Rademacher, personal communication) or it may contain both complex N-linked chains or O-linked chains, as do fetuin (9), human IgA (10,11) and the human erythrocyte membrane sialoglycoprotein (12). In both simple and complex oligosaccharide chains, the same core structure exists:

\[ \beta_{1,4} \]
\[ \text{Man} \longrightarrow \text{GlcNAc} \longrightarrow \text{ASN} \]

a. Simple oligosaccharides.
Oligosaccharide chains which contain only mannose and N-acetylglucosamine residues are certainly not simple, and are more appropriately called 'high-mannose' chains.
Ovalbumin is an example of a glycoprotein containing a high-mannose oligosaccharide and consists of a series of glycopeptides varied in composition from:

$(\text{Man})_5(\text{GlcNAc})_2\text{-Asn}$

to $(\text{Man})_6(\text{GlcNAc})_4\text{-Asn}$.

From Kobata's laboratory the complete structure of a series of ovalbumin glycopeptides has been reported (13,14,15). The remarkable feature of the series is that not only do they contain the same β-mannosyl-di-N-acetylchitobiose core structure, but they all have the same branching pattern of the outer chain α-mannosyl residues, for example:

![Figure 1d. An ovalbumin glycopeptide.](image)

**b. Complex oligosaccharides.**

Complex oligosaccharides contain a variable number of outer chains containing galactose and sialic acid linked to a core of mannose and N-acetylglucosamine. A major class of this group of glycoproteins is where the carbohydrate polypeptide linkage is by a N-glycosidic bond between a N-acetylglucosaminyl residue and the amido nitrogen of an asparagine residue in the polypeptide chain (16,17,18,19).
This linkage is not restricted to a specific functional type of glycoprotein and it is present in membrane and secretory proteins, enzymes, and immunoglobulins (Figure 1e)

\[ \alpha (1-3) \quad \alpha (1-6) \]

\[
\begin{array}{c}
\text{Neu5Ac} \\
\text{Gal} \\
\text{GlcNAc} \\
\text{Man} \\
\end{array}
\begin{array}{c}
\text{Neu5Ac} \\
\text{Gal} \\
\text{GlcNAc} \\
\text{Man} \\
\end{array}
\]

\[
\begin{array}{c}
\text{GlcNAc} \\
\text{GlcNAc} \\
\text{GlcNAc} \\
\text{GlcNAc} \\
\text{ASN (297)} \\
\end{array}
\]

Figure 1e.
The biantennary complex oligosaccharide structure found at the Fc region of human serum IgG.
There is usually a common pentasaccharide core containing 2 α-mannosyl residues attached to a β-mannosyl-di-N-acetylchitobiose unit. Terminal sialic acid (Neu5Ac) is usually found in the Fab region of IgG, whereas terminal galactose (Gal) and N-acetylglucosamine (GlcNAc) are more common in the Fc region.

The existence of a common core structure in many of the N-glycosidically linked glycoproteins suggests a common mechanism of synthesis of at least the internal region of the saccharide chain. The sequence of the outer chains is most often:

\[
\text{Sialic Acid} \rightarrow \text{Gal} \rightarrow \text{GlcNAc}
\]

The galactose (Gal) to N-acetylglucosamine (GlcNAc) linkage is usually β1,4. There may be microheterogeneity in the terminal sialic acid (Neu5Ac) residues so that a full complement of two residues is not always present. Furthermore, the linkage to galactose differs in different glycopeptides; in some the sialic acid...
is attached to C-3 of galactose and in others to C-6. Otherwise the sequence and linkages are identical.

It is thought that the distal sugars (GlcNAc, Gal, and Neu5Ac) of the complex carbohydrate chains are added to the core, one sugar at a time, from their respective nucleotide derivatives (2).

The most likely explanation for the non random attachment of various terminal sugars in complex oligosaccharides is that the glycosyltransferases responsible for their addition have specificity requirements, not only for specific sugar residues, but also the three dimensional structural features of the glycosaccharides. Thus, a structure (Figure 1e) may be a suitable acceptor for a galactosyltransferase that adds galactose to the terminal N-acetylglucosamine residue, but may not act as an acceptor for sialytransferases until the second galactosyl residue has been added. The structures found therefore not only supply clues about their biosynthetic assembly, but also provide the substrates needed to define the specificity of various glycosyltransferases.

C. Glycoprotein synthesis.

i. Introduction.

An explanation for the observation that the core regions are in many instances identical may be provided by the demonstration that both simple and complex-type oligosaccharides are derived from a single high-molecular-weight lipid-linked oligosaccharide (Figure 1f) (20).

![Figure 1f. The proposed structure of the lipid-linked oligosaccharide precursor in glycoprotein synthesis.](image)

The core oligosaccharide is preassembled on the lipid carrier, dolichol phosphate, and only after assembly is complete is the oligosaccharide transferred to the protein acceptor.
ii. Dolichol.
The dolichols are a member of the polyisoprenol family (21) and are found in a variety of eukaryotic tissues (22). Dolichol is probably the longest natural hydrocarbon made up of a single repeating unit.

$$\text{CH}_3 - \text{O - P - O - CH}_2 - \text{CH}_2 - \text{CH} - \text{CH} = \text{C} - \text{CH}_2 - (\text{CH}_2 - \text{CH} = \text{C} - \text{CH}_2)_{17} - \text{CH}_2 - \text{CH} = \text{C} - \text{CH}_3$$

Figure 1g.
The structure of dolichol phosphate.
The length approaches 100 Å, although a helix-like form that is only a little over 70 Å in length has been reported (23).

Figure 1h.
A proposed sequence for the processing of complex oligosaccharides on the endoplasmic reticulum within the golgi. Filled-in squares represent N-acetylglucosamine, circles galactose and diamonds sialic acid, open circles represent mannose and triangles fucose.

When the final product is a complex-type oligosaccharide, additional mannose residues are clipped, and the sugar residues, which form the outer branches are added. A proposal for the sequence of processing is shown (Figure 1h) (23), in which initial major clipping and then major addition are demonstrated.
iii. Oligosaccharide assembly.

![Diagram of oligosaccharide assembly]

**Figure 1i.**
The sequential assembly of the core oligosaccharide unit on the endoplasmic reticulum.
Specific transferases catalyze the synthesis of the activated core oligosaccharide, whilst attached to dolichol phosphate (Dol-P), which is then transferred en bloc to a specific asparagine residue of the growing polypeptide chain.

Dol=dolichol, GlcNAc=N-acetylgalactosamine, Man=mannose, Glc=galactose, GDP=guanidine diphosphate.

In the initial step of oligosaccharide assembly, GlcNAc-1-PO4 is transferred from UDP-GlcNAc to Dol-P (Figure 1i). The second step involves elongation of isolated GlcNAc-P-P-Dol to the disaccharide-lipid, β-GlcNAc-GlcNAc-P-P-Dol, by addition of a GlcNAc unit from UDP-GlcNAc. Enzymatic synthesis of the trisaccharide-lipid containing one unit of mannosyl β-linked to the distal GlcNAc unit of GlcNAc-GlcNAc-P-P-Dol involves direct transfer of a
mannosyl unit from GDP-mannose. Man-P-Dol can serve as a mannosyl donor in the next step, elongation of $\beta$-Man-$\beta$-GlcNAc-GlcNAc-P-P-Dol to oligosaccharide-P-P-Dol. The newly synthesised oligosaccharide chains are then transferred to their peptide backbone.

The first report on an oligosaccharide containing galactose, mannose, and N-acetylglycosamine, linked by a pyrophosphate bridge to dolichol (Dol), had the following structure, where $x$ may be as great as 14.

$$(\text{Glc})_2-(\text{Man})_x-(\text{GlcNAc})_2-P-P-Dol$$

iv. Oligosaccharide transfer.

a. To endogenous membrane proteins.
Experiments using $[^{14}\text{C}]$-mannose and $[^{3}\text{H}]$-GlcNAc-labelled oligosaccharide-lipid (24) show that both labels are transferred to the protein, suggesting that the oligosaccharide chain is transferred en bloc. Further evidence for this is obtained by the identical properties of the reduced oligosaccharide chain released from the protein, with the reduced oligosaccharide prepared from oligosaccharide-P-P-dolichol (25).

b. To exogeneous soluble proteins.
In N-glycosidically linked glycoproteins the asparagine (Asn) attached to the carbohydrate chain is invariably part of the tripeptide sequence

$$\text{Asn-X-Ser- or -Asn-X-Thr}$$

where $X$ represents an amino acid (26,27). Not all proteins containing this sequence are glycosylated. Some are never glycosylated, others, for example bovine pancreatic RNA, are found in un-glycosylated (RNase A) and glycosylated (RNase B) forms (28). Sequence data of tripeptides found in eukaryotic glycoproteins and proteins have been analysed (2,27) and about one third were found to exist in a glycosylated form which seems to be more likely when $x$ is not aspartate.

Apart from this finding, the amino acid ($x$), however, probably has little influence over glycosylation of asparagine.

The secondary structure of the peptide segment around the carbohydrate peptide linkage has also been analysed (29) and in more than half, the glycosylated asparagine residues are localized at a $\beta$-turn in the polypeptide chain. It is not
surprising, therefore, that glycosylated tripeptides containing these amino acids are found in more disordered regions in the final protein. Glycosylation therefore seems to be regulated by the occurrence of the ASN-XSer/Thr sequence at a point accessible to the glycosyltransferase enzymes of the lipid-linked pathway. Glycosylation is thus controlled essentially by higher orders of structure imposed by the primary sequence of the protein, and one could hypothesise that glycoproteins arise during evolution by point mutations that produce an acceptor tripeptide sequence in a region of a protein which becomes accessible to the transferases of the lipid-linked pathway.

v. A model for glycoprotein synthesis.

It is known that secretory proteins are synthesised on the rough endoplasmic reticulum, and there is evidence that secretory proteins pass through the membrane at the endoplasmic reticulum as they are synthesised and are deposited in the cisternal space upon polypeptide chain completion (30). It has been shown that insertion of several secretory proteins into the membrane involves the formation of an initial hydrophobic sequence of amino acid residues termed a "signal peptide" (31,32). In addition, it has also been shown that viral membrane glycoproteins are also synthesised on the rough endoplasmic reticulum (33,34), with the glycoprotein inserted through the membrane in a similar manner, so that its carbohydrate chain is located in the cisternal space (33). Protein glycosylation is thought to be temporally related to translation, since polypeptides that are newly completed, but still attached to tRNA, are already glycosylated (35,36). On this basis, one would predict that the enzymes involved in saccharide-lipid synthesis and transfer of the oligosaccharide chain from dolichol pyrophosphate to the protein would be found in the rough endoplasmic reticulum, where translation takes place. To support this it has been shown that the overall lipid-linked system is highly enriched in the rough endoplasmic reticulum (37), and that mannose is added to the extension peptide of procollagen within the lumen of the rough endoplasmic reticulum (38). All these findings can be brought together as follows:
In the first stage (Figure 1j), the nucleotide derivatives of N-acetylglucosamine (open circles) and mannose (closed circles), synthesised in the cytoplasm, react with dolichol phosphate in the rough endoplasmic reticulum to form the monoglycosyl lipids. Further reactions, leading to formation of oligosaccharide-P-P-dolichol could occur within the hydrophobic environment of the membrane. Glycosylation would occur when a -Asn-X-Ser/Thr- tripeptide sequence has been translated and is oriented so that it can interact with both the other substrate, oligosaccharide-P-P-dolichol, and the membrane-associated oligosaccharide transferase. Upon completion of translation, secretory proteins no longer remain associated with the membrane, whereas membrane proteins, because of the presence of sequences of hydrophobic amino acids, remain attached to the rough endoplasmic reticulum. The only distinguishing feature between membrane and secretory glycoprotein being their primary structure.

In the second stage, the secretory glycoproteins move into the smooth endoplasmic reticulum-Golgi complex. Similarly, the membrane glycoproteins move into this compartment, perhaps by lateral diffusion in the plane of the membrane system. Here glycoproteins could be modified by addition of fucose and the distal sugars, N-acetylglucosamine, galactose, and sialic acid to the basic carbohydrate chain.
Figure 1k.
Oligosaccharide synthesis within the golgi. Stages 3 and 4. The open and closed circles refer to monosaccharide molecules.

In the third and fourth (final) stage (figure 1k), secretory vesicles are formed, and these fuse with the plasma membrane, the secretory glycoproteins being deposited outside the cell, and membrane glycoproteins oriented on the plasma membrane, with the carbohydrate chain on the external surface.

The explanation for the development of processing on the polypeptide may be related to the generation of diversity amongst complex oligosaccharide structures. Thus, many primitive organisms such as yeast and fungi contain high-mannose oligosaccharides, while complex-type oligosaccharides appear to have developed at a later time in evolution. This observation suggests that high-mannose oligosaccharides are the ancestors of complex-type oligosaccharides and that processing represents a mechanism for synthesizing complex-type oligosaccharides without the necessity for developing a second lipid-linked oligosaccharide pathway.

vi. Regulation of synthesis.
Alterations in cellular glycoproteins may be of significant biological importance and there are a number of potential regulatory sites. These include alteration in the level of:

a. Initial glycosyl donors, the sugar nucleotides;
b. The glycosyl acceptor, dolichol phosphate;
c. The transferases that catalyze these reactions, and hence the overall synthesis of oligosaccharide-P-P-dolichol,
d. The oligosaccharide transferase; and
e. The acceptor apoprotein.
Specific control of N-glycosidically linked glycoproteins by regulation of the level of UDP-GlcNAc is unlikely since this sugar is found in other types of glycoproteins and in proteoglycans. In contrast, mannose is uniquely found in N-glycosidically linked glycoproteins and in keratan sulphate type I in higher eukaryotes, so that it is possible that the control of the level of GDP-Man might affect the level of glycoprotein synthesis. Little is known about alterations in level of sugar nucleotides. Similarly, there is little information about regulation of the glycosyltransferases involved in assembly of oligosaccharide-lipid. Dolichol pyrophosphate fluctuations may also be a potential regulatory area for study.

vii. Inhibition of synthesis.
Inhibitors of glycosylation are important tools for studying the biological role of the carbohydrate moiety of glycoproteins, as well as the process of glycosylation itself. The most commonly used general inhibitors have been glucosamine and 2-deoxy-D-glucose (39). In addition, the related compounds 2-fluoro-2-deoxy-D-glucose and 2-fluoro-2-deoxy-D-mannose have also been shown to block glycosylation in eukaryotic cells (40,41) it has also been demonstrated that the antibiotics tunicamycin and bacitracin inhibit the lipid-mediated glycosylation process. These antibiotics may be of greater research value than the sugar analogues, since their effects appear to be more specific.

2. Glycosyltransferases.
A. Introduction.
These enzymes catalyse the reaction that can be generally expressed as:

\[ \text{Nucleoside diphosphate sugar} + \text{R-OH} \rightarrow \text{Sugar-O-R} + \text{nucleoside disphosphate} \]

The glycosyl donor is a sugar nucleotide, and the acceptor may be a monosaccharide, oligosaccharide, glycopeptide, glycoprotein or, a glycolipid. Some of the glycosyltransferases transfer a sugar residue not to the hydroxyl group of a sugar, but to the hydroxyl group of an amino acid such as serine, threonine, or hydroxylysine. The linkage between sugar and phosphate in all sugar nucleotides involves the anomeric carbon of the sugar (C-1 of D-glucose, D-galactose, D-mannose, L-fucose, N-acetyl-D-glucosamine, and N-acetyl-D-
galactosamine and C-2 of sialic acid); this same anomer carbon becomes glycosidically bonded to the acceptor hydroxyl group.

In mammalian tissues, the sugar nucleotides of D-glucose, D-galactose, D-mannose, N-acetyl-D-glucosamine, and N-acetyl-D-galactosamine are all α-linked, whereas L-fucose is β-linked to GDP. Some sugars, for example, L-fucose and N-acetyl-D-glucosamine, appear to be transferred from sugar nucleotide to acceptor primarily by a single inversion of anomer configuration, i.e., the anomer configurations of L-fucose derived from GDP-β-L-fucose and of N-acetyl-D-glucosamine derived from a UDP-α-N-acetyl-D-glucosamine are α and β, respectively in most mammalian complex carbohydrates. Other sugars, however, are found in complex carbohydrates in both α and β-linkages. For example, D-galactosamine is found both α and β-linked and the α and β-N-acetyl-D-galactosaminyltransferases, carrying out these incorporations, all use α-linked sugar nucleotides (42,43).

B. Glycosyltransferase assay.

i. Assay techniques.

Although it is possible to assay lactose synthetase and other glycosyltransferases by spectrophotometric methods (44), the most accurate and commonly used assay method, especially with crude enzyme preparations, involves measuring the transfer of radioactive sugar from sugar nucleotide to acceptor. Crude enzyme preparations may contain nucleotide pyrophosphatase, CMP-sialic acid hydrolase, glycosidases or non-specific phosphatases. Thus, a typical enzyme incubation may contain several radioactive components, i.e. sugar nucleotide, sugar phosphate, free sugar, and product. Further, the product may be due to the transfer of radioactive sugar to both exogenous and endogenous acceptors; the endogenous acceptors may be small or large oligosaccharides, glycoproteins, or glycolipids or even non-carbohydrate-containing contaminants. It is therefore important that proper assay conditions are established for every individual experiment.

Several techniques have been developed for the separation of product from the reaction mixture:

a. High voltage paper electrophoresis.

A reliable, but tedious, method, is the use of high-voltage paper electrophoresis in 1% sodium tetraborate at pH 9.0 (45). Sugar nucleotide migrates rapidly towards the anode and is cleanly separated from product which usually remains
at or near the origin. Sugar phosphate also migrates rapidly towards the anode, but at a slightly different rate than the sugar nucleotide. Free sialic acid, galactose, mannose, and fucose migrate towards the anode either because of an inherent negative charge or because of borate complex formation. However, N-acetylhexosamines do not complex borate efficiently, and these materials are removed from the origin of the paper by descending chromatography with 80% ethanol. The product remaining at or near the origin can then be counted directly on the paper by liquid scintillation techniques. It is important to count several centimetres of paper near the origin since many products, even proteins, migrate slightly and tend to smear; counting a large segment of paper indicates whether there is efficient separation of the product from other radioactive materials. Endogenous acceptor assays should be routine and the appropriate corrections applied. If the endogenous acceptor is a protein or a lipid, the resulting product will remain at the origin and interfere with the exogenous acceptor assay. The endogenous acceptor activity can either be subtracted or, if a lipid, can be washed out of the paper with organic solvent. The high-voltage electrophoresis assay is a useful assay for work with crude enzyme preparations.

b. Precipitation and filtration.
A more rapid assay involves the precipitation of product with trichloroacetic acid or orthophotungstic acid (46,47) followed by filtration on a glass fibre filter. The filter is then washed with either trichloroacetic acid or orthophosphotungstic acid followed by organic solvents, then dried, and counted. This method is only useful with high-molecular-weight acceptors, as all low-molecular weight radioactive compounds are removed by this procedure. Endogenous lipid acceptors do not interfere since the resulting products are removed with the organic solvent wash. When sialytransferases are assayed by this method, the entire procedure should be carried out with ice-cold solutions, due to the acid lability of bound sialic acid. Crude enzyme preparations can also cause problems, especially when working with mucin acceptors, due to clogging of the filters and resultant trapping of low molecular weight radioactive compounds in the filter.

c. Dowex column.
Another rapid method involves passage of the reaction mixture through a small column of Dowex 2 X8 (Sigma), 200-400 mesh, in the chloride form, equilibrated in water (48). Sugar nucleotide and sugar phosphate are retained on
the column while radioactive product and free sugar pass through the column and are counted. The "endogenous acceptor" control therefore will contain not only endogenous acceptor activity but also free sugar from sugar nucleotide breakdown. The amount of free sugar formed may not be constant from incubation to incubation, especially with crude enzyme preparations containing nucleotide pyrophosphatase, non-specific phosphatases, glycosidases acting on radioactive product, and other hydrolytic enzymes. The assay is fast and convenient but should be used only when glycosyltransferase preparation is relatively free of interfering hydrolytic enzymes.

d. Gel filtration.
Gel filtration using columns of Sephadex G-50 (fine) equilibrated with buffer and 0.01% sodium azide is a useful technique (49). High molecular weight product elutes before low molecular weight radioactive compounds can be readily counted. The assay is rapid and effective but cannot be used with low molecular weight acceptors.

e. Paper chromatography.
Reaction mixtures can be subjected to paper chromatography on ordinary filter paper (50,51) or on DEAE ion-exchange paper (52). The latter procedure is effective only with low-molecular-weight acceptors since sugar nucleotide and sugar phosphate remain at the origin and product must migrate away from the origin for the assay to work. The paper chromatographic approach is reliable but slow. It is useful in determining the nature of low-molecular-weight radioactive derivatives formed in an incubation, but not as a routine procedure in enzyme purification or kinetic experiments.

f. ELISA.
Alkaline phosphatase conjugated bovine GTase has been used to quantitate the amount of GTase protein in an inhibition assay, and is reported to be sensitive to 30ng (53).

ii. Assay Conditions.
It is important to establish saturating conditions for both acceptor and sugar nucleotide and to maintain saturation throughout the course of the reaction. Acceptor concentrations must be individually optimised since excess acceptor may cause substrate-dependent inhibition (51,54,55,56).
Most glycosyltransferases (with the exception of sialytransferases) require divalent cation for activity. Intracellular glycosyltransferases are membrane-bound and require some sort of detergent and/or ultrasonic or homogenisation treatment for expression of optimum activity; such treatment is usually not required for extracellular glycosyltransferases such as are present in milk, colostrum, serum, amniotic fluid, etc.

The glycosyltransferases show a high degree of specificity for the sugar being transferred, for the base component of the sugar nucleotide, and for the sugar residues at the non-reducing end of the acceptor. It is likely that a separate transferase is required for every known sugar-sugar linkage; this concept has been called the "one linkage-one enzyme" hypothesis (57). The hypothesis was originally proposed on the basis of indirect evidence for the existence of multiple transferases (58) but has received strong support from work on highly purified enzymes (49,59).

C. Glycosyltransferases involved in elongation of N-acetyllactosamine linked oligosaccharide

i. General concepts.
As described (1.B.ii), N-glycosidically linked oligosaccharides occur in two major forms, the oligomannoside form and the N-acetyllactosamine form and it appears that synthesis of possibly all N-glycosidically linked oligosaccharides may be initiated by the transfer of oligosaccharide from a dolichol pyrophosphate oligosaccharide to protein.

It is not known why some oligosaccharides remain in the oligomannoside form while others are processed down to the Man₃GlcNAc₂Asn core and subsequently elongated to N-acetyllactosamine-type oligosaccharides.

ii. Classification.

a. N-Acetylglucosaminyltransferase.
N-Acetylglucosaminyltransferase was first reported in goat colostrum (59) and catalyzes the following reaction.

\[
\text{UDP-}\alpha\text{-GlcNAc} + \text{Man-}\alpha\text{-R } \rightarrow \text{GlcNAc-}\beta\text{-Man-}\alpha\text{-R} + \text{UDP}
\]

It has been shown, (60,61,62) that enzyme activity, determined by the use of high-molecular-weight acceptors, such as glycosidase-treated α1-acid
glycoprotein, is due to at least two N-acetylglucosaminyltransferases designated, GlcNAc-transferase I and II. GlcNAc-transferase I attaches GlcNAc preferentially to the Man-\(\alpha 1,3\)-arm of the structure:

\[
\text{Man} \quad \alpha_1,3 \\
\text{Man-}\beta_1,4\text{-GlcNAc-R} \\
\text{Man} \quad \alpha_1,6
\]

GlcNAc-transferase II attaches GlcNAc to the Man-\(\alpha 1,6\) terminus of the product of GlcNAc-transferase I.

b. Fucosyltransferases.

When fucose occurs in N-glycosidically linked oligosaccharides, it is found only on N-acetyllactosamine structures, not oligomannoside structures, and linkage is, almost invariably, to the most internal GlcNAc residue, i.e., the residue attached to asparagine (2). GDP-fucose; \(\beta\)-N-acetylgalactosaminide fucosyltransferase catalyses the reaction leading to:

\[
\text{GlcNAc} \quad \beta_1,2\quad \text{Man} \quad \alpha_1,3 \\
\text{Man} \quad \beta_1,4 \\
\text{Man} \quad \alpha_1,6 \\
\text{GlcNAc} \quad \beta_1,4 \\
\text{GlcNAc-ASn}
\]

The fucosyltransferase can act on the products of either GlcNAc transferase I or II, but cannot act until at least one GlcNAc residue has been incorporated into the Man\(3\) GlcNAc\(2\) Asn core structure. Thus, GlcNac-transferase I controls the addition of fucose into the core.

Some of the structural features of N-glycosidically linked oligosaccharides can therefore be examined by the specificity of the fucosyltransferase. It is, however, as yet unclear why some N-acetyllactosamine structures contain the fucose-GlcNAc-Asn structure (e.g. human IgG) while others do not (e.g. human \(\alpha 1\)-acid glycoprotein), possibly the appropriate fucosyltransferase is absent.
c. UDP-Gal: N-Acetylglcosaminide galactosyltransferase.  
Galactose is almost invariably located at the same position in all N-glycosidically linked oligosaccharides, as part of the sequence:

Galactose-β(1,4)-GlcNAc-β-Man-α-Man-β-GlcNAc  

Galactose may be the terminal non-reducing sugar or may be penultimate to a sialic acid. β1,6-GlcNAc sequences have also been described. Many tissues have been shown to contain an enzyme or enzymes capable of catalysing the following reaction:

\[
\text{UDP-α-D-Gal + GlcNAc-β-R} \rightarrow \text{Gal-β(1,4)-GlcNAc-β-R + UDP}
\]

R can be -H or oligosaccharide, glycopeptide, or glycoprotein.

UDP-Gal: N-Acetylglcosaminide galactosyltransferase (GTase) activities are found inside most cells as membrane-bound enzymes, localised to the Golgi apparatus (63) and in a soluble form in milk, amniotic fluid, cerebrospinal fluid, saliva, urine, colostrum, and serum (57). The milk galactosyltransferase is equivalent to the A protein of lactose synthetase (64); the B protein is α-lactalbumin, which has no known enzymatic activity. Lactose synthetase A protein has a low affinity for glucose, although it can synthesise lactose at very high glucose concentrations. In the presence of α-lactalbumin, the affinity of the A protein for glucose is greatly enhanced (64). Milk A protein in the absence of α-lactalbumin can transfer galactose from UDP-Gal to GlcNAc to make N-acety lactosamine and can also transfer galactose to larger β-N-acetylglucosaminides. It is interesting that the UDP-Gal: β-N-acetylglucosaminide galactosyltransferases in liver, serum, and other tissues can make lactose in the presence of exogenous α-lactalbumin (44,56).

The half-life of human GTase is approximately 20 hours (65) and purified bovine milk galactosyltransferase has a specific activity of about 5.6μmole/min per mg protein at 23°C (66). The enzyme contains two major catalytically active forms with molecular weights estimated at 55,000-59,000 and 42,000-44,000 (67), and there is evidence to suggest that the smaller form is derived from the large form by proteolysis due to a trypsin-like milk protease which may be identical to plasmin (68), although work with HeLa cell GTase indicates the presence of 2 precursor polypeptides (45,000 & 47,000) which are
translated by different messenger RNAs (69). Both forms are glycoproteins and contain about 10-15% carbohydrate (67). The gene encoding human GTase is thought to be located on chromosome 9 (70).

The interaction of bovine milk galactosyltransferase and α-lactalbumin is a unique example of enzyme modification (71). The interaction requires manganese (Mn\(^{2+}\)) and either glucose or N-acetylglucosamine. It appears that Mn\(^{2+}\) is always the first ligand to react with the enzyme, followed by UDP-galactose to form the enzyme-Mn\(^{2+}\)-UDP-Gal-acceptor complex which dissociates to release UDP and product. The addition of α-lactalbumin complicates the kinetics and serves to lower the Km for glucose about 1000-fold (71). Physiologically, lactose synthesis occurs only in the mammary gland at parturition and during lactation, when α-lactalbumin is formed presumably in response to a hormonal signal.

The physiological role of the galactosyltransferases in tissues, other than lactating mammary gland, is almost certainly the elongation of N-glycosidically linked oligosaccharides. It is evident that no galactose can be incorporated into the core, until at least one GlcNAc is incorporated, which implies GlcNAc-transferase I controls the addition of galactose residues.

d. Sialytransferase.

In N-glycosidically linked oligosaccharides, sialic acid is always found at the non-reducing terminus in an α-linkage to a subterminal galactosyl residue. Sialytransferase catalyzes the following general reaction:

\[
\text{CMP - sialic acid} + \text{Gal-β-R} \rightarrow \text{Sialyl-α-Gal-β-R} + \text{CMP}
\]

Many tissues have been shown to contain membrane-bound enzymes capable of transferring sialic acid to various low-molecular-weight acceptors such as lactose and N-acetyllactosamine and to high-molecular-weight acceptors such as sialidase-treated α1-acid glycoprotein or fetuin (57); soluble forms of these enzymes have been described in goat, bovine, and human colostrum (72) and in pork and human serum (55, 73). Sialytransferase is thought to compartmentalised within cytoplasmic vesicles (63).

Data suggests the existence of at least four sialytransferases (74). Two enzymes are required for attaching sialic acid in α2,3 and α2,6 linkages to a galactosyl residue of N-linked oligosaccharide and a further two enzymes are required for making similar attachments in O-linked oligosaccharides.
iii. The physiology of glycosyltransferase in normal and disease states.

Normal galactosyltransferase function has been investigated (75,76) and is associated with sperm-egg binding (77), cell-cell recognition (78,79) and normal embryonic development (80). Sperm GTase is a major sperm surface receptor for binding the zona pellucida during fertilisation (81).

Studies have suggested that a fraction of serum galactosyltransferase activity, may correspond to blood group B activity, (82) and galactosyltransferase activity in serum of blood group A and O subjects has been described which adds galactose to acceptors with terminal N-acetylgalactosamine (83). Human platelets have demonstrable galactosyltransferase activity but are not likely to contribute significantly to serum enzyme activity (84).

Interestingly, elevation of serum glycosyltransferase activity has been reported to correlate with the presence of tumour tissue (85,86) and similar findings have been reported in plasma galactosyltransferase in patients with metastatic carcinomas from breast, colon, and ovary (87,88,89). Serial determinations in patients with ovarian carcinoma show a correlation between galactosyltransferase activity and clinical status (90). It is likely that the increase in glycosyltransferase comes from tumour cells themselves (91,92) and these findings may be considered evidence for altered glycoprotein synthesis in tumour cells, which may include cell surface membrane glycoproteins.

Perhaps the best evidence that a particular glycosyltransferase enzyme is released from tumour cells comes from the isolation of a unique isoenzyme of galactosyltransferase (Galactosyltransferase II, GTII) in sera of cancer patients (88,93). It is highly homologous in composition and kinetics, yet quite distinct, varying in size, amino acid and carbohydrate content. A larger series has supported these observations, but patients with severe alcoholic hepatitis, other liver diseases, active coeliac disease and chronic renal failure also have detectable GT-II activity (86,94). In this context, it is interesting to note that depressed levels of serum galactosyltransferase has been reported in patients with cystic fibrosis (95).

It has also been proposed (96), that galctosyltransferase plays a role in tumour metastases, as GT-II bound to normal tissue may provide a receptor for malignant cell binding. Changes in carbohydrate moieties of glycoconjegates that occupy the cell surface have been noted in numerous transformed cells, as has the appearance on the cell surface of enzymes involved in glycosylation (96). Galactosyltransferase is present in abundance on the surface of BALB/c
3T12 cells, and plays an integral role in the controlled growth of these cells (97).

It has been observed that the removal of sialic acid from certain cell surface glycopeptides of transformed or malignant cells, causes these glycopeptides to have similar chromatographic properties to those derived from non-malignant cells (94). It is suggested that a specific sialytransferase in transformed or malignant cells may be responsible for sialic acid-enriched glycopeptides (98,99).

Tumours may therefore possess glycoproteins containing altered carbohydrate content and the discovery of carcinoembryonic antigen (91) and the recognition that many tumour-associated antigens are glycoproteins, enhances the concept of tumour specific alterations in glycoprotein structure and biosynthesis.

3. Oligosaccharide function.

A. Introduction.

That the carbohydrate side chains play a role in the conformation of glycoproteins has been shown by the three-dimensional structure of an intact human immunoglobulin deduced from X-ray crystallographic data (100). Using a human IgG cryoglobulin, the complete quaternary structure of the IgG molecule, including placement of the complex oligosaccharide chains attached at Asn-297 of each heavy chain, was obtained.

![Space filling view of an IgG molecule. The large black spheres represent the individual hexose units of the complex carbohydrate.](image)

Figure 11.
The hexose units of the carbohydrate (black spheres), and the branched oligosaccharide chain can be seen to wrap partly around the Cγ2 domain and prevent it making contact with the Cγ1 domain. The fact that the carbohydrate occupies a fixed position in the molecule and plays a major role in the interaction of the Fc and Fab regions suggests that its absence would alter the conformation of IgG and perhaps its functional properties as well. Support for this comes from glycosidase digestion experiments to remove carbohydrate from rabbit and sheep red blood cell IgG antibody (101). The antigen combining site of the Fab region is unaffected in the sugar-depleted IgG, however, the Fc region is affected, since it is no longer able to interact with Fc receptors either on monocytes or on cytotoxic lymphocytes, and has lost its capacity to mediate complement fixation.

Oligosaccharide units of the viral glycoproteins also have a major effect on the properties of these proteins. Tunicamycin has been used to block the glycosylation of influenza virus haemagglutinin (102) resulting in degradation of the haemagglutinin precursor by intracellular proteases. This suggests that the oligosaccharide units of the haemagglutinin may protect it against non-specific cleavage.

Immunoglobulin M is glycosylated at five sites in the constant region of the μ heavy chain, and position 402 is thought to be analogous to ASN 297 in the IgG molecule (103). Differences in glycosylation are thought to determine whether the secreted form of IgM is monomeric or polymeric (104). N-linked oligosaccharides are present on the α H chain of IgA and are essential for intracellular stability and normal secretion of the molecule (105).

Terminal galactose predominates in the native murine IgE N-linked carbohydrate chains, and are essential for Ig secretion (106). Increased glycosylation in the V region of human monoclonal anti-dextran antibodies increases binding affinity (107), whereas deglycosylation of rabbit Fc increases its immunoreactivity towards monoclonal antibodies (108).

Non-enzymatic glycosylation of IgG and diabetes mellitus has been associated with alterations of antibody function (109), but this is probably not related to a breakdown in immune regulation (110).

B. Immunoglobulin G glycosylation.

i. Introduction.

The three-dimensional structure of an oligosaccharide (glycoform) (Figure 1m) (111) and its monosaccharide sequence give great potential for
information reception and transmission. The glycoform is heterogenous, and hence polypeptides can become diversified by associating with various glycoforms. This may be significant as each structure may be associated with a particular cellular function. Disease associated changes in the incidence of individual glycoforms, or the generation of new glycoforms, may therefore have profound effects, and changes in oligosaccharides could in some cases contribute directly to disease pathogenesis. A comparative analysis of the N-glycosylation of particular glycoproteins provides a novel means of investigating particular disease processes and disease associations and some interesting data has recently been published concerning these associations (112,113,114,115).

Figure 1m.
The three dimensional structure of IgG, showing the bi-antennary oligosaccharide chains associating with each other between the Cγ2 domains in the Fc region.

All normal IgG isotype antibodies are glycoproteins carrying predominantly N-linked oligosaccharides (112,116). Human serum IgG carries, on average, 2.8 N-linked oligosaccharides, of which 2.0 are located in the Fc at the conserved N-glycosylation site of Asn 297 (Figures 1m&n). The additional oligosaccharides are located in the variable region of the light and heavy chains, with a frequency and position dependent on the occurrence of the amino acid sequence Asn/X/Ser(Thy). Approximately 30 different biantennary oligosaccharides are associated with total human serum IgG (112). These are
distributed non-randomly between the Fab and Fc. Characteristics of Fc N-glycosylation include the absence of disialylated structures, a low incidence of monosialylated ones (10%), a low incidence of cores carrying a 'bisecting' GlcNAc, and the absence of galactose on the α1-3 arm of at least one of the oligosaccharide chains in each Fc (112,117). Fab N-glycosylation is characterised by a high incidence of di- and monosialylated structures, and of cores with the 'bisecting' GlcNAc residue. IgG oligosaccharide heterogeneity is not the result of studying a polyclonal population, since similar findings are observed on analysis of myeloma and hybridoma IgG (112).

ii. IgG N-glycosylation analysis.

Serum IgG N-glycosylation patterns can be determined using the following methods:

a. **Compositional analysis** using gas-liquid chromatography (118). This technique only gives an estimate of the total galactose and GlcNAc and not the degree of biantennary chain glycosylation (119,120,121,122). High pressure liquid chromatography has also been used (123) and can be linked to a mass spectrophotometer (124).

b. **Sequence analysis** using hydrazine to release IgG-associated oligosaccharides, followed by digestion of the oligosaccharides with exogenous enzymes of defined specificities (112). This technique gives accurate estimations of oligosaccharide chain glycosylation.

c. **Lectin binding assays**, using the properties of Bandeiraea simplicifolia lectin and Ricinus communis agglutinin to bind GlcNAc and Galactose (125). This method incorporates a 'Dot-Blot' technique to quantitate the amount of biotinylated lectin binding to the degraded IgG molecule. The optical density changes are reported as ratios of the two lectins, thus measuring fluctuation of both galactose and GlcNAc over the whole IgG molecule. In isolation, this method would therefore not be ideal if IgG-Fc changes are required, but it would appear that the major changes are likely to take place at the Fc region as this method has been shown to accurately reflect data obtained from sequence analysis, but is accomplished in a much shorter time (ie: 1 week vs 1 month).

N-glycosylation analysis has also been performed using 3 serial lectin columns (Aleuria aurantia, RCA 120-WG003 and phytohaemagglutinin) (126).

d. **Anti-GlcNAc monoclonal antibodies**, can be used to bind to agalactosyl oligosaccharide chains (127), but provides information only on the relative incidence of agalactosyl oligosaccharides.
iii. IgG glycosylation and age.

Immunological competence of both B (128,129) and T (130) cells is known to vary with age. The IgG-associated structures from individuals varying in age from 1-70 years have been analysed with respect to glycosylation, sialylation, outer-arm N-acetylglucosamine content and degree of core-substitutions (113). The incidence of structures with both outer-arms terminating in N-acetylglucosamine was found to first decrease with age from >30% to 20% at 25 years and then increase continuously to reach a level of 40% by 70 years of age (Figure 3c). This corresponds to a nearly 4-fold increase in those serum IgG molecules whose Fc may be presumed to be totally devoid of galactose between 25 and 70 years of age. Differences between the sexes in galactosylation of IgG oligosaccharides are not significant, and nor are there any differences in sialylation and core substitution. There are, however, a limited but significant variation in the incidence of glucosyl oligosaccharides within an age group. Interestingly, the occurrence of 1 out of 3 galactose residues does not vary with age, whilst 2 out of 3 galactose residues varies inversely to G(0)% (no galactose residues). This data serves to correct the original hypothesis that IgG glycosylation was decreased in patients with OA (112) and make it imperative that all data relating to oligosaccharides and IgG is age-related.

Variation in glycosylation differs from previously reported age-dependent molecular parameters (131,132), such as glucose mediated cross-linking of proteins (132), in so far as it appears to be a continuous and natural process applied to a serum protein, of relatively short half-life, in a manner independent of the level of that protein.

An explanation for age dependent glycosylation for IgG may be due to a naturally age-related expression of β-galactosyltransferase or that certain clones of B-lymphocytes, differing with respect to their N-glycosylation capacity, dominate at different developmental stages of the immune system (133). This may account for the increased frequency of idiopathic paraproteinaemias during ageing (134).

The high incidence of agalactosyl IgG in serum of young children (113) may indicate a particular N-glycosylation capacity in B-lymphocytes in these children and it is interesting that in children with juvenile rheumatoid arthritis, the high incidence of agalactosyl structures found during the early years after birth is maintained and does not return to normal until periods of sustained remission (114).
Some human B lymphocytes, which secrete rheumatoid factor, are CD5 phenotype positive, and are found both in patients with rheumatoid arthritis and in cord blood from healthy offspring (135,136). It has been suggested that CD5+ B-lymphocytes may also be characterised by a particular N-glycosylation capacity (113). Paradoxically the increased frequency of autoantibodies and rheumatoid factor are characteristic of older humans (137). These data raise the possibility that one lesion in rheumatoid arthritis may be an accelerated 'ageing'-process within the B-lymphocyte compartment of affected individuals.

iv. IgG glycosylation in rheumatoid arthritis, mycobacterial infection, Crohn's disease, other diseases and pregnancy.
When N-glycosylation patterns of serum IgG (Figure 1e.) are studied differences are readily apparent between age-matched normal individuals and those with rheumatoid arthritis (112,138). Novel oligosaccharides are not found in rheumatoid serum IgG, but an increased number of oligosaccharide moieties whose outer arms lack galactose and terminate in N-acetylglucosamine is seen (112). There is therefore a shift in the population of IgG glycoforms towards those with a higher content of agalactosyl-oligosaccharides.

From a study of other autoimmune rheumatological conditions, chronic inflammatory disorders, and diseases with known infectious aetiology (115), it emerges that only juvenile chronic arthritis, tuberculosis, Crohn's disease, and SLE with Sjögren's syndrome are invariably associated with an elevated G(0)%", whereas the other similar disease (eg: ankylosing spondylitis, infective endocarditis, ulcerative colitis, HIV, Reiter's syndrome and psoriatic arthritis) are not. Other reports suggest similar IgG glycosylation abnormalities in leprosy, when associated with erythema nodosum leprosum (139) and Lyme disease (140). Furthermore, G(0)% correlates well with clinical score in patients with adult rheumatoid arthritis and also with disease activity (114). To date, intestinal mucin is the only other glycoprotein reported to have a reduced galactose component in RA patients when compared to normal individuals (140a).

The finding of increased agalactosyl IgG in patients with mycobacteria tuberculosis is consistent with the suggestion that mycobacteria, or autoantigens which cross-react with them, might be implicated in the pathogenesis of rheumatoid arthritis (141).

Immune-complexes containing self-associated IgG have been identified in the serum of tuberculosis patients (142), and clearance studies have shown that IgG from these patents behaves similarly to that present in rheumatoid arthritis
Inflammatory arthritis associated with acute Mycobacterium tuberculosis (MTB) was described as early as 1897 (144), and arthritis has been reported after BCG immunotherapy (145). Sera from many patients with TB have autoantibody (146) and rheumatoid factor activity (142). Antigenic resemblance between mycobacterial cell wall glycolipid (Figure 10.) and DNA has been suggested, and antibodies to DNA and mycobacteria have been found to cross-react (147,148).

An association between HLA-DR phenotype and skin test response to mycobacterial antigens has been reported (149,150). The HLA-DR4 phenotype, which is closely associated with RA in Caucasians (151), also confers a higher responsiveness to MTB antigens in patients with leprosy and to tuberculin in patients with RA. Those RA patients with HLA-DR7 haplotype (protective) showed low skin test responsiveness to mycobacteria. Decreased levels of antibody binding to mycobacteria in those patients with rheumatoid arthritis who carry the relatively protective DR2 or DR7 haplotype has also been reported. Furthermore, antibody levels to crude mycobacterial antigens in sera from patients with RA showed significant correlation with HLA-DR haplotypes (152) and raised IgA levels to crude mycobacterial antigens, together with both raised IgA and IgG levels against the 65kD and 71kD antigens have also been reported in RA patients (153).

The immune response of RA patients to an acetone-precipitated fraction of M. tuberculosis that cross-reacts with human cartilage has been assessed (154) and RA patients have more pronounced T-cell responses to the antigen than patients with degenerative joint disease or healthy controls. However, this may be due to a class II (DR4) antigen association (155). But, interestingly, arthritogenic and arthritis 'protecting' T-cell clones have been generated from rats with adjuvant-induced arthritis (156). Intriguingly, they have been shown to recognise both MTB, and antigens present in human cartilage and synovial fluid. Furthermore, studies with synthetic peptides have shown that the relevant peptide sequence to which these clones react has sequence homology to a 65kd stress protein and human cartilage peptidoglycan (141,157,158).

In 1932 Crohn, Oppenheimer and Ginsberg defined "regional ileitis" as a clinical and pathological entity distinct from intestinal TB, but they did not rule out a role for mycobacteria in its pathogenesis (159), which is still unclear. Although the production of agalactosyl-IgG link RA, TB and CD, the diseases are themselves markedly dissimilar. Perhaps the site of agalactosyl IgG production is important, or may be additional differences in Fab glycosylation.
are important. Most likely, however, agalactosyl-IgG is simply one piece of the mosaic of factors resulting in RA and Crohn's disease.

In this respect a comparative analysis of IgG from serum and synovial fluid (112) has shown that in patients with rheumatoid arthritis, the level of G(0) in the synovial fluid is higher than in serum. This data may indicate that agalactosyl IgG is synthesised within the joint.

Pregnancy is known to influence the RA disease pattern (160) and the carbohydrate composition of IgG from serum of pregnant women with RA has been determined (161) and found to contain more galactose and sialic acid than normal IgG, whereas fucose, N-acetylglucosamine, and the total carbohydrate content were unchanged. These data suggest temporal compensation of the RA associated undergalactosylation of IgG in female patients with RA during pregnancy, a period during which remission of the disease is often observed. These data are also supported by observations of reduced clinical score in RA patients when pregnant associated with normal G(0)%. The clinical score and G(0) subsequently reverse during the postpartum period (162).

However, the changes in IgG glycosylation in pregnancy may also be due to the altered hormonal state. Oestrogens and prolactin (163,164) do influence glycoprotein glycosylation and the association between autoimmune diseases such as RA and steroid hormones has been well described (165,166).

v. Carbohydrate-dependent functions of IgG.

a. IgG-Fc glycosylation.

An increase in the population of IgG glycoforms terminating in N-acetylglucosamine may have pathological consequences. Carbohydrate-dependent functions of the Fc moiety of IgG all involve interactions with cellular-bound receptors and although agalactosyl and deglycosyl IgG molecules retain the properties of normally glycosylated IgG in the binding of antigen, protein A, IgM rheumatoid factor, and, also with respect to Cl activation (167), there is complete loss of binding to monocyte and macrophage Fc receptors (167,168) and reduced Clq binding (169). The ability to induce cellular cytotoxicity is also reduced (168), complexes with antigen fail to be eliminated rapidly from circulation (168), and feedback-immunosuppression is lost (170). It is also known that, within the total IgG population, a range of affinities for monocyte Fc receptors exists independent of subclass, and which correlates with Fc N-glycosylation (171), which would indicate that not only the degree, but also the nature of Fc N-glycosylation is of biological relevance.
b. IgG-Fab glycosylation.

Univalent antibodies, formed by Fab glycosylation, have enhanced cytotoxic properties (172) and a terminal mannose situated on one Fab arm is thought to act as a ligand in complement activation (173). Immune complex aggregation may also be influenced by Fab glycosylation. The presence of sialic acid confers cold agglutinin properties on certain monoclonal antibodies (174). Pathological effects consequent on an agalactosylation of IgG may thus be caused by alterations in interaction with monocyte Fc, and immune complex formation.

c. Immune complex formation and glycosylation.

IgG auto-sensitisation may play a pivotal role in the pathogenesis of rheumatoid arthritis (175). Immune complexes are formed through the binding of the IgM, IgG, or IgA rheumatoid factors to the constant region domains of IgG molecules and the immunogenic site on IgG is thought to be localised to the Fc moiety (176). There is, however, no evidence for amino acid changes in IgG-Fc, but immune-complex formation in rheumatoid arthritis may involve both Fab N-glycosylation and agalactosyl structures in the Fc (133). Crystal structure of Fc may provide some insight into the molecular mechanism of IgG self-association (112), and the crystal structure shows that each N-linked oligosaccharide in the Fc can interact with the protein surface of the Cy2 domain, principally via the sialic and galactose segment of the Man α1-6 arm (Figures 1m&n.).

IgG self-association could occur either by insertion of a Fab-linked oligosaccharide from another IgG molecule into this vacant site through the interaction of the affected IgG with either naturally occurring or induced anti-G1cNAc antibodies, or through interaction of the affected IgG with antibodies induced against the peptide or peptide-oligosaccharide epitopes previously masked by the glycosylated oligosaccharide. An analogous situation is found with the pharmacological use of recombinant human granulocyte macrophage colony stimulating factor, where antibodies are formed in patients against the recombinant protein only when they are exposed due to a lack of O-linked oligosaccharides (177).
d. N-acetylglucosamine (GlcNAc) receptors.
GlcNAc receptors may be functionally important in the recognition of peptidoglycan structures of bacterial cell walls (178), in tumour surveillance by natural killer (NK) cells (179), and in macrophage recognition of cells undergoing programmed cell death (apoptosis) (180). The membrane CD3 receptor, which is expressed on phagocytic and NK cells, has been shown to bind GlcNAc-containing glycans (181) and endotoxin, possibly via the N-acylated glucosamine-containing moiety (182). Furthermore, certain macrophage functions have been shown to be activated by N-acetyl-chito-oligosaccharides (183). IgG molecules presenting an abnormally high concentration of exposed non-reducing terminal GlcNAc residues may therefore provoke immunopathological changes as a consequence of interaction with cells bearing receptors for the GlcNAc epitope (127). This 'molecular mimicry' between antigens of infectious agents and host tissues has been used to explain the induction of autoimmune disease by microbes (141).
4. Diseases involved in this study.

A. Rheumatoid Arthritis (RA).

i. Introduction.
RA is a multisystem disorder of unknown aetiology, causing immune dysfunction characteristically resulting in articular inflammation and extra-articular abnormalities, which form part of the criteria for diagnosis (184). Genetic susceptibility is well defined, but the disease in its present form was rare before the 19th century.

ii. Epidemiology.
It is estimated that 1-3% of the UK population are affected with RA, with incidence increasing with age but peaking between 30-40 years. Females are 2 to 3 times more susceptible, but sex difference is not apparent over 65 years. Genetic susceptibility associated with the gene products of the major histocompatibility system is well defined. The B lymphocyte HLA class II alloantigen DR4 being present in about 70% of patients with serpositive RA. There are further associations with other HLA DR gene products, for example, DR1.

iii. Clinical manifestations.
Clinical progression in RA is variable, but is characterised by exacerbations and remissions and with season and climate affecting some patients. Distinct patterns of disease can be observed, however, with disease presentation occurring either as a single attack, that may not be repeated, as repeated brief attacks with recovery (palidromic), or as repeated severe attacks and residual joint damage.
About 70% of all patients develop chronic disease, but only 30% of those with palidromic RA will go on to develop persistent disease. Overall 5-10% of patients will develop severe disease.
Rheumatoid arthritis characteristically affects certain synovial joints in a symmetrical fashion. The following are the most commonly affected:
HANDS (especially the metacarpophalangeal and proximal phalangeal joints), WRISTS, ELBOWS, HIPS, KNEES, FEET, CERVICAL SPINE.
RA may also cause extraarticular manifestations and the following organs are commonly involved:
SKIN (Nodules, vasculitis, ulcers), NERVES (neuropathy, nerve entrapment, cervical myelopathy), EYES (Sicca syndrome, scleritis, episcleritis), LUNGS (Effusion, nodules, interstitial fibrosis, obliterative bronchiolitis), HEART (Peri, endo, & myocarditis, conduction defects), KIDNEYS (Proteinuria/nephrotic syndrome), MARROW (Anaemia).

The following syndromes are associated with RA:

a. Sicca syndrome (secondary Sjögren's syndrome) is the association of dry eyes and dry mouth (keratoconjunctivitis sicca) with RA.

b. Rheumatoid vasculitis is an uncommon extraarticular manifestation of RA and may present with dermal infarcts, especially at the nailfolds, a peripheral sensory neuropathy, a mononeuritis multiplex, which may cause foot-drop, or cerebral and cardiac vessel involvement and occlusion.

c. Felty's syndrome is rare and consists of the triad of rheumatoid factor positive RA, splenomegally and neutropenia. Synovitis is usually severe, and there is an increased incidence of extraarticular features, including weight loss, leg ulcers and infections.

iv. Treatment.

The aim of clinical treatment is to maintain joint function, prevent or correct deformities and pharmacologically suppress the immunological mediators of inflammation. A combined physical, medical and surgical approach to the care of the patient with RA is beneficial in tackling these problems.

The following drugs are commonly used in the treatment of RA:

a. First line agents: Aspirin
   Other Nonsteroidal anti-inflammatory drugs. (NSAIDs)
   eg: Ibuprofen, Naproxen, Piroxicam.

b. Second line agents: Sulphasalazine (SASP).
   Gold salts.
   D-Penicillamine.
   Chloroquine.

c. Third line agents: Corticosteroids
   Azathioprine
   Cyclophosphamide
   Metotrexate
B. Systemic lupus erythematosus (SLE).
i. Introduction.
SLE is a multisystem disease of unknown aetiology, in which cellular and tissue damage may be caused by autoantibodies and immune complexes, producing a wide spectrum of clinical manifestations.

ii. Epidemiology.
90% of those developing the disease are female, and the usual age of presentation is between 25 - 35 years. The average female incidence is approximately 1:250 in black races, 1:4000 in caucasians and 1:1000 in chinese.

iii. Pathogenesis.
It is thought that the basic abnormalities are the production of pathogenic autoantibodies and immune complexes and the failure to suppress them.

iv. Aetiology.
There is probably a multifactorial aetiology to SLE, and there are a number of hypotheses:

a. Genetic.
High concordance in monozygotic twins
Familial tendency
HLA associations: HLA DR2, DR3, and probably DQw1
Inherited deficiencies in complement components present in some patients

b. Environmental.
Viruses, bacteria, UV light

c. Sex hormonal factors.
Oestrogen enhances antibody responses, and there is a greater incidence in females.

d. Abnormal humoral and cellular responses

e. Inadequate clearing of antibodies and immune complexes.

v. Clinical manifestations.
Disease manifestations are determined, in part, by the presence of certain antibody populations, their targets, and the patients' ability to correct these abnormalities. Hence, one system or many may be involved.
The following are common clinical manifestations:

a. **Systemic:**
   - Fatigue, malaise, fever, anorexia, weight loss, and nausea.

b. **Musculoskeletal:**
   - Arthralgia, myalgia,
   - Arthritis is symmetrical and mainly involves the metacarpophalangeal and proximal interphalangeal joints, wrists, and knees. Deformity is unusual and may be due to capsular laxity. Soft tissue rheumatism is common (ie: tenosynovitis).

c. **Cutaneous:**
   - One of the classical features is the malar "butterfly" rash over the cheeks and bridge of nose, or face, which is exacerbated by UVlight.
   - Alopecia (patchy). Will regrow, except in discoid lupus.
   - Vasculitis - subcutaneous nodules, ulcers, purpura, infarcts of skin.
   - Discoid lupus - Lesions are circular with erythematous rim, raised, and scaly with follicular plugging and telangiectasia. There is depigmentation of the dermo-epidermo-junction which is due to central scarring and degeneration of the basal layer.

d. **Renal:**
   - Depending on the population studied between 30-50% of patients have clinical nephritis on urinalysis (haematuria, casts and proteinuria).
   - Most lesions are caused by immune complex deposits, and in mild nephritis there is either no change histologically or proliferation confined to the mesangium.
   - Glomerular nephritis may occur, with the following changes:
     1. **Focal proliferative**
     2. **Membranoproliferative**
     3. **Diffuse proliferative**
     - Groups 1 & 2 and group 3 have an 85% and 70% 5 year survival respectively.

e. **Nervous system:**
   - CNS involvement in SLE is an important cause of morbidity and the brain, meninges, spinal cord and peripheral nerves may all be involved. There is consequently a wide spectrum of neurological manifestations including mild mental dysfunction, seizures, depression, anxiety, hemiplegia, cerebellar ataxia and cranial nerve lesions.
f. Vascular:
Thrombosis may occur due to vasculitis, antibodies (anti-cardiolipin or the 'Lupus anti-coagulant') and immune complex deposition.

g. Haematological:
Amaemia
1. Nonproliferative (75%) or a Coomb’s positive haemolytic anaemia.
2. Leukopenia.
3. Mild thrombocytopenia.

h. Cardiopulmonary:
Pericarditis - effusions occur, but tamponade is uncommon.
Myocarditis - can cause arrhythmias and cardiac failure.
Endocarditis (Libman-Sacks).
Pleurisy and effusions are common.
Pneumonitis - fever, dyspnoea and cough, which rarely leads to fibrosis.
Adult respiratory distress syndrome and intraalveolar haemorrhage is rare.

i. Gastrointestinal:
Non-specific symptoms are common.
Vasculitis may result in pain, vomiting, diarrhoea and lead to perforation.
Pseudo-obstruction is recognised by radiography which shows dilated loops.
Acute pancreatitis may occur.

j. Ocular:
Retinal vasculitis causes infarcts (cytoid bodies).
Conjunctivitis, episcleritis, and optic neuritis.
Sicca syndrome is also associated.

vi. Laboratory findings:
Characteristic autoantibodies:
a. are not specific for SLE, and may be found in normal individuals, especially during illness ie: acute viral infections.
b. in high levels ANA and anti-dsDNA, together with the presence of immune complexes, usually reflect disease activity.
Low levels of complement may also reflect disease activity as may a raised ESR. False positive VDRL, abnormal coagulation (PTT), and rheumatoid Factor (30-50%) are also found.
vii. Diagnosis.
The diagnosis of SLE rests upon the recognition of a constellation of clinical and laboratory findings, and criteria for classification has been devised (185). Briefly, this is based upon the presence of characteristic skin rashes, arthritis, together with disordered renal, neurological, haematological, and immunological systems.

viii. Treatment.
The following are examples of the current pharmacological treatment of SLE.

a. NSAID: Arthralgias, fever, serositis, myalgia
b. Antimalarials: Dermatides and lupus arthritis
c. Sunscreens, topical or intralesional steroids: Skin involvement

ix. Prognosis.
Complete remissions are rare and the overall 10 year survival is 71%. Infection and renal failure are the main causes of death.

C. Tuberculosis.
i. Introduction.
Mycobacterium tuberculosis (MTB) was first described by Robert Koch in 1882 (186). The species is, like M.leprae, an obligate parasite with no free-living saprophytic forms. But, unlike M. leprae, it is a facultative intracellular parasite, capable of either intracellular or extracellular existence. It can also cause infection in many wild, farm, and domestic animals. Two main species are recognised, MTB and Mycobacterium bovis. MTB by far the most important of the mycobacterial pathogens, accounting for almost all cases of human tuberculosis.

MTB is well adapted for parasitisation, having characteristics enabling it to survive host defences for many years. For example, it is slow growing and has a high lipid content to its cell wall (acid fast). Its optimum temperature range is narrow, nutritional requirements exacting, and it is a strict aerobe. It can also lie dormant for many years. Bacillary death is difficult to achieve once infection has taken place.
ii. Morphology.

Tubercle bacilli are long, curved, often beaded or banded rods, 4µm or more in lengths and about 0.5µm in diameter. They tend to form long cords and are acid fast on Ziehl-Neelsen staining. Mycobacterial cells are rich in lipids, and their biological effects and adjuvant activity are related to these lipids. The superficial lipids, (mycosides, cord-factor, sulphotides) principle ones are mycolic acids, glycolipids, phospholipids, and mycosides, all of which are found predominantly in the highly complex cell wall. Superficial to and within the wall structure are free lipids, including mycosides (which are glycolipids or peptidoglycolipids), trehalose 6,6'-dimycolate (cord factor), and sulpho-lipids. Tubercle bacilli do not produce exotoxins, endotoxins or extracellular enzymes noxious to the host and the precise mechanism of their virulence is not at all clear.

iii. Pathology and immunology.

The basic response to tubercle bacilli is cellular and involves phagocytosis of the organism by macrophages. Within days a typical concentric tubercle has formed, with epithelioid and giant cells surrounded by macrophages. Before activation, by being stimulated by sensitized T lymphocytes, macrophages can probably only kill a minority of virulent bacilli. When activated, macrophages have a greater capacity to destroy bacteria, but they behave non-specifically, and

Figure 10.
A diagramatic representation of the highly complex mycobacterial cell wall.

Cell membrane

Superficial lipids, (mycosides, cord-factor, sulphotides)

Mycolic acid

Arabinogalactan

Peptidoglycan

59
indeed increase the resistance of the host to a large number of micro-organisms. Delayed hypersensitivity is also induced by tubercle bacilli infection, and can cause a considerable amount of local tissue damage.

iv. Epidemiology.
Tuberculosis has been a major affliction of man since ancient times. During the industrial revolution tuberculosis epidemics were common, but the disease has declined steadily, probably because of improved nutrition and social conditions, leading to better host resistance.
Most people in Europe and North America were, until recently, infected by tubercle bacilli at some time in their lives. In contrast, among the younger generation, infection is now uncommon. In developing countries, however, infection is the rule. Notification rates have declined rapidly since the introduction of effective chemotherapy in the UK. Currently the rate is declining at approximately 10 per cent per annum. A survey of notifications for 1978-79 in England showed an estimated annual notification rate of 18 per 100,000, the rate for respiratory tuberculosis being 13, and for non-respiratory 5. Among patients of the Indian sub-continent origin respiratory disease is 30 times more common than in white patients and non-respiratory disease is 80 times more common.
It has been argued that hypersensitivity and acquired immunity are separate immune responses directed against different components of the mycobacterial cell. Certainly hypersensitivity is not responsible for acquired immunity. Probably the two responses involve different lymphokines, and so different populations of T cells.

v. Treatment.
In technically advanced countries, where the incidence of tuberculosis is low, medical services relatively plentiful, and drug costs generally not a limiting factor, a daily regimen of isoniazid and rifampicin for 9 months, with ethambutol or streptomycin for the first two months, is currently recommended.

vi. Diseases caused by non-tuberculosis mycobacteria.
The mycobacterial environmental saprophytes which are capable of facultative parasitism include some important human pathogens, notably, M. kansaii, M. avium, including the intracellular variant, and M. scrofulaceum. The parasitism of these mycobacteria is often opportunistic, involving the colonisation of tissues already damaged or rendered susceptible by necrosis or
by some other disease, but it is important to realise that many of them can also cause disease in previously healthy subjects.

D. Crohn's disease.

i. Introduction.
Crohn's disease is a chronic granulomatous inflammatory disease of the gastrointestinal tract, affecting any part, frequently in discontinuity. The first clear description of the disease affecting the terminal ileum (regional ileitis) was given by Crohn, Ginzburg, and Oppenheimer in 1932 (158).

ii. Epidemiology.
Crohn's disease is well recognised in Europe, Scandinavia, North America, and Australia, but it is rarely seen in India, tropical Africa and South America, which may be due to difficulties of diagnosis, especially as intestinal tuberculosis is relatively common. There has been a marked increase in the incidence and prevalence of Crohn's disease in Europe and Scandinavia since 1950, rising from 2.8 per 100,000 in 1958 to 7.2 per 100,000 in 1971 in England and Wales.
The reasons for the changing patterns of incidence are not clear, and it is possible that the changing incidence may result from an infective or environmental factor. Crohn's disease occurs in all age groups but it is rare in early childhood and most commonly affects young adults. There is no marked sex difference, and no association with social class or occupation.

iii. Genetics.
There is a definite familial incidence of the disease, varying from 6-15 per cent. However, there is no clear mode of inheritance and there is no established association with HLA types. Discordance for the disease in identical twins suggests that environmental factors may be operating.

iv. Aetiology.
Although pathogenesis is still unclear it is interesting to note that homogenates of Crohn's disease tissues have been shown to induce unusual tissue reactions and granuloma formation in the intestine of rabbits (187,188) and in the foot pads of mice (189,190). Slow growing cell-wall defective Ziel-Neelson staining organisms have been isolated from mesenteric lymph nodes draining the lesions of patients with Crohn's disease, and recent work has confirmed the presence of such organisms in tissue from inflammatory bowel disease has been
observed (191,192,193). These organisms were not grown in sufficient quantity for characterisation, but several groups have cultured an assortment of conventional mycobacterial species from Crohn's disease tissue (191,192,193). Various mycobacterial species are common nevertheless, in biopsies of normal bowel wall (193) and there seems little justification for linking these with Crohn's disease other than the intriguing observation of raised G(0)% in these patients.

v. Immune mechanisms.
Serum immunoglobulins and complement levels are usually normal in these patients, although they may be raised in active disease. Inhibitors of leucocyte mobility may be present in serum, and the absolute number of circulating T lymphocytes may be reduced. The histological features suggest that a cell-mediated response may be involved. Lymphocytes from some patients appear to be sensitised to colonic epithelium, or coliform antigens, and circulating lymphocytes may be cytotoxic to colonic epithelium, but this may be secondary to inflammation. Antigen antibody reactions may be another important effector mechanism as suggested by increased consumption of complement and the presence of circulating small immune complexes. Impairment of suppressor cell activity during active disease has been found which might account for the increased circulating antibody titres to dietary and bacterial antigens.

vi. Pathology.
Macroscopically the bowel is thickened and frequently stenosed. The serosal surface may be inflamed and the mesentery becomes oedematous. The earliest macroscopic lesion on the mucosal surface is an aphthoid ulcer. In areas of more severe disease, deep fissuring ulcers occur in the oedematous and inflamed mucosa. Histologically, the inflammation is transmural and consists principally of lymphocytes, histocytes and plasma cells. Granulomata are found in only 65 per cent of patients and they occur with increased frequency the more distal the disease. Quantitative histology and enzyme studies have suggested that the whole of the gastrointestinal tract is abnormal in patients with Crohn's disease, even though only one segment may be overtly involved at any one time.
vii. Clinical features.
Patients with CD generally present with symptoms of abdominal pain, a change in bowel habit and, as the disease progresses, sequelae to chronic bowel inflammation; such as fistulae and strictures. There are a number of extra-bowel manifestations of the disease, apthous ulceration and arthritis for example. The arthritis is commonly HLA B27 associated, assymmetrical, rheumatoid factor negative and has features similar to ankylosing spondylitis. In the UK arthritis occurs in about 20% of patients with CD.

viii. Diagnosis.
The diagnosis is made by clinical, biological and serological features, but may not be straightforward. The main differential diagnosis is tuberculosis, ulcerative colitis, malignancy and infection (eg: yersinia and actinomycetes). There is no satisfactory method of assessing activity of the disease and this poses a major clinical problem. Symptoms such as fever or continuing weight loss are obvious indicators, but severe disease can be present in the absence of any major symptoms. Laboratory evidence of activity includes reduced serum albumin levels and increased C-reactive protein concentration.

ix. Treatment and prognosis.
The management of Crohn's disease involves medical therapy, surgical treatment, and nutritional support. Crohn's disease is only treated if it is causing symptoms. Patients are never cured of Crohn's disease and they are subject to relapses of their disease and to recurrence following surgical resection. In general, the majority of patients with Crohn's disease have a good prognosis with a mortality of only about twice that expected. Considerable morbidity can be expected, but this will be intermittent and the overall quality of life should be good.
5. The MRL/lpr autoimmune mouse

The murine strain MRL was developed as a by a series of crosses involving strains C57BL/6J, C311/Di, AKR/J and LG/J and the strain contains an estimated 0.3% 12.1% 12.6% and 75.0% of the above genomes respectively (194).

The spontaneous autosomal recessive mutation lpr (lymphoproliferation) produces massive generalised lymph node enlargement in MRL mice by 8 weeks of age. Females die at an average age of 17 weeks and males at 22 weeks with immune complex glomerulonephritis.

An increase in proportion and absolute numbers of thymic-derived lymphocytes is described and the thymus is marginally enlarged with an atrophic cortex. Juvenile thymectomy suppresses the development of the abnormal phenotype. Hypergammaglobulinemia and marked increases in both IgG1 and IgG2a are characteristic. Antinuclear antibodies, including anti-double stranded DNA, anti-Sm and thyroglobulin autoantibodies develop.

MRL +/+ mice do not develop the generalised lymphoproliferative syndrome. Females die at an average of 73 weeks of age and males at 92 weeks with chronic glomerulonephritis. Necrotising arteritis and reticulum cell neoplasms are found, as are antinuclear antibodies.

The congenic MRL lpr/lpr and +/+ mice were initially proposed as the model of choice for SLE. However, these mice, also spontaneously develop inflammatory arthritis in the hind limbs, may possess serum IgG and IgM rheumatoid factors and develop antibodies to Type I and II collagen, thus exhibiting morphological and immunological similarities to RA, which would suggest that the MRL/lpr mouse may serve as a reasonable animal for RA (195). Recent studies indicate histomorphological similarities with human rheumatoid synovium (196), and also features common to Sjörgren's syndrome (197).
CHAPTER 2.

CHARACTERISATION OF LYMPHOCYTIC GALACTOSYLTRANSFERASE

1. Methods used in the estimation of human lymphocytic galactosyltransferase (GTase) activity in this study.

A. Isolation of B and T cell enriched populations from peripheral human blood.

Peripheral blood was obtained by venesection and, when required, depleted of monocytes by incubation at 37°C with carbonyl iron, followed by separation with a magnet. Peripheral-blood lymphocytes were then separated by means of 'Ficoll-Hypaque' (198). Cell purity was assessed by May-Grunwald-Giemsa staining, the lymphocytes accounting for at least 95% of the total population. The B cells and thymus-derived T cells were further separated by rosetting with neuraminidase-treated sheep red blood cells. Assessment of cell purity by rerosetting showed at least 95% rosettes in the T-cell population and no more than 10% rosettes in the B-cell population.

The lymphocytes were incubated at 37°C for 1 h in balanced salt solution containing 10% fetal calf serum, then resuspended in 100 mmol/l tris-HCl buffer (pH 6.8) containing 0.1% 'Triton-X100' (Sigma) and 0.1% 2-mercaptoethanol (BDH), frozen and thawed twice, and homogenised in a glass homogeniser. The homogenate was incubated at 37°C for 1 h, and the supernatant, after centrifugation at 10,000 rpm for 10 min, was assayed for enzyme activity.

B. Determination of lymphocytic galactosyltransferase activity
(Figure 2a).

The standard incubation medium for the assay contained:

- 0.5 mmol/l ATP (Sigma).
- 20 mmol/l manganous chloride (BDH).
- 100 μmol/l UDP-galactose (Sigma).
- 1μCi UDP-[3H] galactose (Amersham International).
- 1 mg ovalbumin (Sigma)

in 100 mmol/l tris-HCl buffer (pH 6.8), to a total volume of 100 μl,
Figure 2a.
A standard curve relating the amount bovine GTase (µU) to the amount of transferred [³H] galactose (cpm.10³).

after addition of 20 µl enzyme extract. The mixture was incubated, in duplicate, for 2 h in a shaking water bath at 37°C. The reaction was stopped by the addition of 2 ml 1% phosphotungstic acid in 0.5 mol/l hydrochloric acid. The precipitate was filtered onto Whatman GF/A glass microfibre filters, washed, dried, and counted. Protein concentration was estimated (Bio-Rad Laboratories) and the enzyme activity either expressed as cpm/mg protein or by calculation of mMol/hr/mg protein from a standard curve obtained using bovine galactosyltransferase (Sigma).

C. Protein Estimation (Figure 2b).
Protein estimation between the values 1-5 µg/ml was achieved using the 'Bio-rad' protein assay kit.
This assay relies upon dye binding to produce a differential colour change of an acidic solution of Coomassie Brilliant blue G-250 in response to various concentrations of the protein (199). This causes a shift in the absorbance maximum from 465 to 595 nm.
Between 20-40 µl of either 1:5 or 1:10 dilution of enzyme extract, in 100 mmol/l tris-HCl buffer (pH6.8), was taken, in duplicate, and made up to a total volume of 75 µl in a 96 well ELISA plate. 300 µl of Bio-rad reagent (1:4 dilution in water) was added to each well.
Figure 2b.
BSA protein estimation.
Standard curves of 3 experiments using the 'Bio-rad' assay system.

Standard curves were constructed for each estimation using concentrations of bovine serum albumin (BSA) ranging between 1-5 µg/ml. (Figure 2b)
Absorbance was subsequently read at 595 nm. A reproducible linear relationship was achieved using this method.

2. Units of GTase activity.
In initial experiments, GTase activity was expressed as nMol/hr/mg protein, by comparison with a commercial standard preparation of bovine GTase (Sigma); one unit of commercial preparation being the amount of GTase required to transfer one mole of galactose per hour. In later experiments, the units of GTase activity were expressed as counts per minute/mg protein, as the commercial preparation was not found to be a reliable standard, due to product variability and degradation during storage of the aqueous preparation. Excess UDP-galactose and constant specific UDP-galactose radioactivity enables relative activities to be compared in each experiment. In this way inter-laboratory enzyme activity comparison is possible as the relationship between counts per minute and specific activity can be determined.
3. What incubation reagents are required to maximise detection of GTase activity?

A. Introduction.
Reagents necessary for a GTase catalysed reaction have been suggested by various reports (90,200) and all were incorporated in the protocol (2.B), except that experimentation with various buffers, other than tris-HCl, (eg: Cacodylate and HEPES) was not carried out. To test their requirement, and to maximise the detection efficiency of the reaction, the catalytic reaction was carried out in the absence of certain basic ingredients. Counts per minute were corrected for background activity (ie: due to endogenous acceptors).

B. Method.
5.10^6 mixed lymphocytes were taken from a normal individual (JSA), GTase prepared and its activity determined in duplicate, as described. Paired catalytic reactions were carried out in the absence of:
- UDP-galactose.
- ATP.
- Manganous chloride.
with a one hour incubation time.

C. Results (Table 2.1).
In the absence of UDP-galactose, ATP and manganese, the enzyme activity detected was reduced by 49% (11012 cpm), 15% (18357 cpm) and 41% (12747 cpm) respectively, when compared to the activity obtained in the presence of all ingredients.

D. Conclusion.
These results demonstrate that excess cold UDP-galactose is required to maximise transfer of the radiolabelled compound. The requirement of manganous ions for the catalytic reaction is also demonstrated, and the addition of ATP appears to confer a slight advantage, probably by inhibiting phosphorylase mediated breakdown of UDP-galactose.
<table>
<thead>
<tr>
<th>Standard incubation mixture</th>
<th>CPM (corrected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>21586</td>
</tr>
<tr>
<td>- UDP-Gal</td>
<td>11012</td>
</tr>
<tr>
<td>- ATP</td>
<td>18357</td>
</tr>
<tr>
<td>- Mn²⁺</td>
<td>12747</td>
</tr>
</tbody>
</table>

**Table 2.1.**

Lymphocytic GTase activity (mean cpm of duplicates) detected, from a normal individual, omitting various ingredients from the enzyme assay.

4. How sensitive is the GTase assay and can this be improved?

**A. Method.**

20μl aliquots of GTase extract, taken from the homogenate of 5.10^6 mixed lymphocytes from a normal individual (JSA), were incubated, in three doubling dilutions, over 1 and 2 hour periods, in duplicate as described.

**B. Results (Table 2.2).**

i. GTase activity from a 3-fold dilution of 5.10^6 mixed lymphocytes is detectable.

ii. A proportional decrease in GTase activity (55623 - 16296 corrected cpm) is observed with decreasing enzyme concentration.

iii. Prolongation of the incubation time to 2 hours results in an increase in corrected cpm by an average of 66% with the 4 dilutions used.
<table>
<thead>
<tr>
<th>ACCEPTOR</th>
<th>DILUTION</th>
<th>1 HR INC.</th>
<th>2 HR INC.</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>1.0</td>
<td>8799</td>
<td>7877</td>
</tr>
<tr>
<td>+</td>
<td>1.0</td>
<td>55623</td>
<td>76126</td>
</tr>
<tr>
<td>+</td>
<td>0.5</td>
<td>37068</td>
<td>74586</td>
</tr>
<tr>
<td>+</td>
<td>0.25</td>
<td>24154</td>
<td>39700</td>
</tr>
<tr>
<td>+</td>
<td>0.125</td>
<td>16296</td>
<td>22205</td>
</tr>
</tbody>
</table>

Table 2.2.
The effects of GTase extract dilution and prolongation of incubation on enzyme activity (mean cpm of duplicates) from 5.10^6 mixed lymphocytes from a normal individual. The endogenous acceptor activity is shown in row 1.

C. Conclusion.
i. The enzyme assay can be used to quantitate GTase activity.
ii. The enzyme assay is sensitive when at least 625,000 cells are used.
iii. Prolonging incubation to 2 hours increases sensitivity and may be useful if small numbers of cells are available. However, if a comparison between dilutions is required then further experimentation would be needed to define the variability in catalytic rates.

5. Does the addition of bovine serum albumin (BSA) prior to filtration improve [3H] galactose-ovalbumin recovery?

This experiment was performed to test whether excess protein (BSA) increased the recovery of the [3H] galactose-acceptor complex by acting as a carrier, after protein precipitation and prior to filtration.

A. Method.
5.10^6 mixed lymphocytes were obtained, from 2 normal individuals, in the usual way and GTase activity assayed in duplicate, over a 1 hour incubation, by the method described. Prior to filtration, 1 mg of BSA was added to the precipitated protein.
B. Results (Table 2.3).
In individuals 1 and 2 a mean decrease by 10% in recovered $[^{3}\text{H}]$ galactose-ovalbumin complex was observed on addition of the BSA carrier protein.

<table>
<thead>
<tr>
<th>Standard incubation mixture</th>
<th>Patient 1</th>
<th>Patient 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Acceptor</td>
<td>8648</td>
<td>7985</td>
</tr>
<tr>
<td>+ Acceptor</td>
<td>25077</td>
<td>23816</td>
</tr>
<tr>
<td>+ Acceptor and BSA</td>
<td>22008</td>
<td>21866</td>
</tr>
</tbody>
</table>

Table 2.3.
The effect of BSA on lymphocytic galactosyltransferase activity (mean counts per minute of duplicates). The endogenous acceptor activity is shown in row 1.

C. Conclusion.
This experiment provided no evidence to suggest that addition of BSA to the precipitated protein in any way increased the sensitivity of the enzyme assay, and simply made the assay more laborious.

6. Can GTase activity be detected using the low molecular acceptor N-acetylglucosamine (GlcNAc) and what is the optimum tracer radioactivity?

A. Experimental objective.
The objective was to carry out two experiments:
a. To determine whether GlcNAc could reliably be used as an acceptor in the catalytic reaction, and to characterise the reaction by varying the amount of bovine GTase and $[^{3}\text{H}]$ UDP-galactose (0.5 and 1.0 $\mu$ci).
b. To determine whether it was possible to decrease the amount of radioactivity used by varying the amount of $[^{3}\text{H}]$ UDP-galactose with a constant amount of bovine GTase (500$\mu$U).
B. Method.
This assay was developed to allow the detection of transfer of radioactive galactose from its UDP carrier to GlcNAc, by washing the complex through a Dowex-1, chloride form, column (Sigma), and allowing non-complexed molecules to attach to the Dowex beads. The activity of flow through was used as an estimate of GTase activity. The stock reaction mixture consisted of the following:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 mMol/l MnCl₂</td>
<td>10 μl</td>
</tr>
<tr>
<td>1mMol/l UDP-gal</td>
<td>10 μl</td>
</tr>
<tr>
<td>5 mMol/l ATP</td>
<td>10 μl</td>
</tr>
<tr>
<td>100 mMol/l Tris-HCl</td>
<td>15 μl</td>
</tr>
<tr>
<td>0.3 Mol/l GlcNAc</td>
<td>20 μl</td>
</tr>
<tr>
<td>UDP-[³H] galactose (1 - 0.125μCi)</td>
<td>5 μl</td>
</tr>
</tbody>
</table>

at pH 6.8.

The incubation time for both experiments was 1 hour and each reaction was carried out in duplicate.
In experiment A, 1.0 and 0.5 μCi [³H] UDP-galactose was used with amounts of bovine GTase varying between 25 and 1000μU.
In experiment B, 500μU bovine GTase was used with amounts of [³H] UDP-galactose varying between 0.125 and 1.0μCi. The reaction was stopped with 30 μl 0.3mol/l EDTA. 1ml of distilled water was added to each reaction tube and transferred to a 25% Dowex-1 (Chloride) column. The UDP-[³H] galactose-N-acetylglucosamine complex was washed through with 5 mls distilled water, collected in aqueous scintillation fluid and counted in a scintillation counter.

C. Results (Figures 2c&d).
i. Experiment A (Figure 2c):
a. Catalytic transfer of galactose to GlcNAc by bovine GTase can be measured using the Dowex column separation technique.
b. There is a linear relationship between GTase activity (corrected cpm) and GTase dilution (31.25 - 1000 μU).
Figure 2c.
The exogenous acceptor activity obtained (mean cpm.10^3 of duplicates), corrected for endogenous acceptor background activity, using increasing amounts of bovine GTase and either 0.5 or 1.0 µCi of ^3H.

ii. Experiment B (Figure 2d):
A linear relationship is demonstrated between the amount of radiolabelled UDP-[^3H]galactose (0.125µCi - 1.0µCi) used per reaction and the GTase activity detected.

Figure 2d.
The relationship between the amount of UDP-[^3H]galactose used in the enzyme assay and the activity obtained.
D. Conclusions.
i. The Dowex column separation method is a sensitive method for detecting the catalytic transfer of radiolabelled galactose to N-Acetylglucosamine.
ii. Decreasing the amount of radiolabelled galactose below 0.5µCi per reaction would limit the sensitivity of the assay.

iii. The activity (cpm.10^3) detected using this method is twice that detected using the filtration method with ovalbumin as an acceptor. This may be due to the relative inefficiency of the filtration method, for example [^3H] galactose-ovalbumin complex may have been washed through the filter. Or, the isoenzymes present in the bovine GTase preparation may be more efficient at transferring galactose to the low molecular weight GlcNAc than the high molecular weight ovalbumin. It is more likely, however, that there is more GlcNAc available for interaction than when ovalbumin is used as the acceptor.

7. Can the GTase catalytic reaction be inhibited by the reaction end product, uridine diphosphate (UDP)?
To confirm that L.GTase activity was being measured, end product inhibition of the catalytic reaction with UDP was investigated.

A. Method.
Supernatant (20µl of 1:4 dilution) from 5.10^6 of mixed lymphocyte homogenate from a normal individual (JSA) was incubated, in duplicate, with the standard catalytic ingredients for the reaction with a high molecular weight acceptor (ovalbumin) for 1 hour, in the presence of increasing concentrations (0.1 - 10 mMol/l) of UDP. 200 µunits of bovine GTase was incubated with similar concentrations of UDP, to test whether both enzymes behaved in a similar fashion.

B. Results (Table 2.4)

<table>
<thead>
<tr>
<th>UDP mMOL/l</th>
<th>0.0</th>
<th>0.1</th>
<th>1.0</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>COUNTS PER MINUTE (Corrected)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human GTase</td>
<td>23816</td>
<td>17565</td>
<td>14981</td>
<td>12372</td>
</tr>
<tr>
<td>Bovine GTase</td>
<td>25682</td>
<td>24943</td>
<td>13973</td>
<td>17179</td>
</tr>
</tbody>
</table>

Table 2.4.
The inhibitory effects of uridine diphosphate (UDP) on human lymphocytic (1:4 dilution) and bovine (200µu) GTase activity (mean cpm of duplicates). The results are corrected for endogenous acceptor activity.
Progressive inhibition of both GTase and bovine GTase, to approximately 48% and 40%, respectively, (with 10 mMol/l UDP) was observed.

C. Conclusion.
UDP is seen to inhibit both GTase and bovine GTase linearly by a similar magnitude, from which it can be inferred that a GTase catalytic system is being detected. That the inhibition is only to just less than 50% with a 10 fold increase in inhibitor was unexpected, and may indicate that fluctuation in intracellular UDP concentration is not a useful feedback control mechanism for GTase.

8. Can GTase be detected in patients with rheumatoid arthritis (RA) and is the assay system appropriate for the study of RA patients?
In order to test these questions, the GTase assay was performed using lymphocytes from RA patients. Inhibition with UDP was tested and bovine GTase was added to the system to test whether the assay system was capable of dealing with increased amounts of GTase or whether it was saturated.

A. Methods.
Lymphocytes (5.10^6) were obtained from three RA patients and GTase extracted. Mean enzyme activity of duplicate 20 μl aliquots was determined over 1 hour with and without the presence of the acceptor, ovalbumin, and with the addition of 100 μUnits bovine GTase and 100 mMol/l UDP.

B. Results (Table 2.5).
i. GTase activity is demonstrated in RA patients using the exogenous acceptor ovalbumin.
ii. [3H]-galactose transfer to endogenous acceptors accounts for background counts ranging from 9630-15795 cpm.
iii. The addition of 100μUnits bovine GTase augmented the recovered activity by between 2 and 5 times.
iv. The reaction was inhibited to below endogenous (background) activity in the presence of 100 mMol/l UDP.
Table 2.5.
Lymphocytic GTase activity (mean cpm of duplicates).
A study to investigate the enzyme catalysed reaction in rheumatoid arthritis in the presence of normal catalytic ingredients and also UDP inhibitor and additional GTase. The endogenous acceptor activity is shown in row 1.

<table>
<thead>
<tr>
<th>Standard incubation mixture</th>
<th>PATIENT 1</th>
<th>PATIENT 2</th>
<th>PATIENT 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>- acceptor</td>
<td>10437</td>
<td>9630</td>
<td>15795</td>
</tr>
<tr>
<td>+ acceptor</td>
<td>41584</td>
<td>47232</td>
<td>55144</td>
</tr>
<tr>
<td>+ 100 mMol/l UDP</td>
<td>260</td>
<td>345</td>
<td>189</td>
</tr>
<tr>
<td>+ Bovine GTase(100 µU)</td>
<td>201725</td>
<td>172328</td>
<td>101642</td>
</tr>
</tbody>
</table>

C. Conclusion.
i. GTase from RA patients has similar characteristics to that from normal individuals, in that it can be detected by similar methods, and can be inhibited by UDP.

ii. Endogenous acceptor presence is demonstrated, which was corrected for in future experiments.

iii. Bovine GTase augmented the extracted GTase by between 2 and 5 times. This would indicate that the assay system was not saturated with the amounts of extracted human GTase used and had the potential to accurately detect greater concentrations of GTase.

iv. Inhibition by UDP infers that GTase was being assayed.

9. The reproducibility of the GTase assay.
The coefficient of variability of 15 samples analysed twice on 2 occasions with 4 weeks separation was 13.4%, whereas that for the same sample analysed 10 times at weekly intervals was 15.7%. When the same sample was analysed 10 times on the same day, however, the coefficient of variability was 4.4%.

These statistics would indicate that the assay is reproducible and reliable, but GTase activity comparison should be made on samples stored for comparable time intervals. As samples were collected and assayed within the same week, any error inherent in the assay due to storage was kept to a minimum.
10. Can plasma GTase be detected in patients with RA?
A similar experiment to that with lymphocytic GTase (section 8) was conducted as a preliminary to determining whether extracellular GTase reflects intracellular (lymphocytic) GTase in RA.

A. Method.
20μl of undiluted plasma was taken from the upper layer of a 'Ficoll' gradient from 3 RA patients and incorporated in the catalytic reaction. The incubation was for 1 hour, and enzyme activity determined, in duplicate, as previously described, using ovalbumin as an acceptor.

B. Results (Table 2.6).

<table>
<thead>
<tr>
<th>Standard incubation mixture</th>
<th>PATIENT 1</th>
<th>PATIENT 2</th>
<th>PATIENT 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>- acceptor</td>
<td>9748</td>
<td>10129</td>
<td>10286</td>
</tr>
<tr>
<td>+ acceptor</td>
<td>23379</td>
<td>22845</td>
<td>31717</td>
</tr>
<tr>
<td>+ 100 mMol/l UDP</td>
<td>374</td>
<td>248</td>
<td>231</td>
</tr>
<tr>
<td>+ Bovine GTase(100μU)</td>
<td>74002</td>
<td>59892</td>
<td>82710</td>
</tr>
</tbody>
</table>

Table 2.6.
Plasma GTase activity (mean cpm of duplicates). A study to investigate the enzyme catalysed reaction in rheumatoid arthritis in the presence of normal catalytic ingredients and also UDP inhibitor and additional GTase. The endogenous acceptor activity is shown in row 1.

i. Background endogenous acceptor presence was detected, contributing counts between 9748 - 10286 cpm.

ii. Plasma GTase activity is detected and this is augmented by between 2 to 3 times with 100μU bovine GTase.

iii. Plasma GTase activity was inhibited to below background levels in the presence of 100mMol/l UDP.

C. Conclusion
i. Plasma GTase appears to have similar characteristics as lymphocytic GTase in this assay system in that it is detectable, augmented by bovine GTase and inhibited by UDP.
11. Do serum levels of GTase reflect lymphocytic activity?

A. Introduction.
Serum GTase activity has been reported to be of value in cancer diagnosis (74-79). Serum GTase presumably originates from a multitude of organs, and probably the majority is from the liver. It was therefore important to determine whether measurement of serum GTase would have any relevance to the study of lymphocytic GTase in RA.

B. Methods.
Six RA patients were studied. GTase activities (mean cpm of duplicates) were estimated, as previously described, over 1 hour, from:
- 20μl aliquots of enzyme extract from a homogenate of 5.10^6 mixed lymphocytes and
- Serum from the same patient.

C. Results (Table 2.7).

<table>
<thead>
<tr>
<th>PATIENT No</th>
<th>COUNTS PER MINUTE (corrected)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LYMPHOCYTES</td>
</tr>
<tr>
<td>18</td>
<td>25705</td>
</tr>
<tr>
<td>19</td>
<td>14873</td>
</tr>
<tr>
<td>20</td>
<td>30192</td>
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</tr>
<tr>
<td>22</td>
<td>30323</td>
</tr>
<tr>
<td>23</td>
<td>46195</td>
</tr>
</tbody>
</table>

Table 2.7.
A comparison of GTase activities (mean cpm of duplicates corrected for background) from human lymphocytes (5.10^6), and serum (ml), to demonstrate that no correlation exists between the 2 measurements.

There was no correlation (r=0.257, p=0.623) between lymphocytic GTase and serum GTase in the six RA patients investigated.
D. Conclusion.
As would be expected, the data from this investigation of six RA patients suggests that serum GTase cannot be used as an estimation of lymphocytic GTase. It would be expected, therefore, that B and T cell activities would be unrelated. Furthermore, low serum levels from one of these sources would be masked by GTase from other sources.
Serum levels of GTase probably result primarily from liver metabolism, and also from cell lysis, which could explain the raised levels observed in malignancies (85-95). Intracellular GTase activity would not be expected to bear any relation to these events.
Also, although in this experiment protein content in the lymphocytic extract was not determined as a known quantity of lymphocytes was used, it was desirable in the future investigations to express accurately GTase activity (ie: as cpm/mg protein) and this would not be possible if serum estimations were used. These observations suggest that there is no advantage in measuring serum GTase levels.

A novel lectin-binding method (125) was employed to determine the amount of serum IgG lacking galactose (G(0)% ) in the patients studied.
Essentially, this involved dot blotting IgG samples onto nitrocellulose and boiling to expose the carbohydrate moieties. The blots were then treated with biotinylated Bandeiraea simplicifolia lectin and Ricinus communis agglutinin, which bind to N-acetylglucosamine and galactose respectively. Binding was detected using streptavidin-hydrogen peroxidase conjugate and developing with chloronaphthol. Optical density ratios were determined with a densitometer and G(0)% determined by comparison with standard IgG of known G(0)% values.
CHAPTER 3.

INVESTIGATIONS OF PATIENTS WITH RHEUMATOID ARTHRITIS.

1. Introduction.
This chapter is divided into two sections:
- A pilot study, upon which all subsequent work is based (section 3).
- The total data collected from patients with RA (section 4).

2. Statistical analysis.
Parametric statistical analyses have been used throughout, except in the pilot study (section 3) where the Mann Whitney non-parametric U-test was used.

3. Pilot study.
A. Experiment 1.
Determination of galactosyltransferase (GTase) activity in whole mononuclear cells and in monocyte depleted mononuclear cells.

i. Patients.
The study population consisted of patients satisfying the revised ARA criteria for a diagnosis of Rheumatoid Arthritis (184) and disease control patients with no clinical nor serological evidence of autoimmune disease (see section 4.A.i for a detailed description of these patients). Disease activity was assessed as inactive/mild (1), moderate (2) or severe (3). (201)
The whole mononuclear cell populations analysed were obtained from nineteen female patients with rheumatoid arthritis (mean age 59.4 years, range 39-82 years) and nineteen age-matched control patients (mean age 58.2 years, range 23-77 years; sixteen female, three male).
The monocyte-depleted mononuclear cell populations came from ten rheumatoid arthritis patients (mean age 53.5 years, range 23-72 years; eight female, two male), and ten age-matched control patients (mean age 50.9 years, range 20-72 years; eight female, two male).

ii. Results (Figure 3a. Reference 202).
The GTase activity of whole mononuclear cell preparations was significantly higher in rheumatoid arthritis patients than in age-matched controls (100.4±8.2 v 61.6±5.0 nmol/h/mg protein.; Mean ±SEM,p < 0.001) whereas in monocyte-depleted mononuclear cell preparations there was no significant difference
between patients and controls (57.8±6.4 v 52.3±6.2 nmol/h/mg protein, Mean ±SEM).

![Graph showing galactosyltransferase activity](image)

**Figure 3a.**
Galactosyltransferase activity (nMol/Hr/Mg protein±SEM) of the whole mononuclear cell preparations.

### B. Experiment 2.
Determination of GTase activity in isolated B and T cell populations.

#### i. Patients.
Isolated B and T cell populations were obtained from seventeen rheumatoid arthritis patients (mean age 62.6 years, range 41-80 years; all female), and eleven control patients (mean age 53.4 years, range 24-75 years; eight female, three male). Nine rheumatoid arthritis patients (mean age 60.3 years, range 41-75 years, range 33-75 years; seven female, two male) were age matched with controls (mean age 59.5 years, range 33-75 years; seven female, two male).

#### ii. Results.
In isolated B cell preparations, GTase activity was significantly lower in rheumatoid arthritis patients than in the controls for the whole group (25.4±7.0 v 81.1±10.6 nMol/Hr/Mg protein; mean ±SEM, p < 0.001) and in the age-matched group 26.2±10.6 v 83.6±12.5 nMol/Hr/Mg protein; mean ±SEM, p< 0.001).
In isolated T-cell preparations, though the GTase activity was significantly lower in rheumatoid arthritis patients than in the controls for the whole group (50.6±14.2 v 81.3±14.6 nMol/Hr/Mg protein; Mean ±SEM, p< 0.05), the difference for the age-matched group was not significant (67.9±24.2 vs 83.5 ±17.8nMol/Hr/Mg protein; mean ±SEM).

4. Analysis of data from the total population of individuals studied.
A. The control population (Table 3.1).

i. Population description.
32 control patients were obtained from those seen in the outpatient clinic at the Bloomsbury Rheumatology Unit and individuals invited to participate in the study. Their mean age was 54.5 years (range 32-78 years) and there were 22 females.

17 members of this population had no complaints of arthritis and were not taking any drugs. 12 members of the population were receiving treatment for osteoarthritis, and one individual was taking a non steroidal anti-inflammatory drug (NSAID). The remaining 3 members of the population were being treated for ankylosing spondylitis (AS), and 2 of these individuals were taking NSAIDs. All the AS individuals were considered inactive on clinical grounds and had ESRs below 15mm/hour.

There was no evidence of autoimmune rheumatic disease in any of these groups.

ii. GTase activity (Table 3.2 & Figure 3b).
Statistics used in analysing this data are the Student's unpaired t-test (one tail) and Pearson coefficient of product-moment correlation.
a. Comparison of control population subgroups.
No difference in GTase activity was observed within the control population as a whole, when divided by sex or the presence or absence of arthritis. Similarly, no differences were observed in subgroup comparisons.

Regression analysis revealed a positive linear relationship between B and T cell GTase activities (Pearson correlation coefficient, r = 0.770, p < 0.001).
<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Sex</th>
<th>Age</th>
<th>B cell GTase</th>
<th>T cell GTase</th>
<th>(G0)%</th>
<th>Disease</th>
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<td>Bailey</td>
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<td>75</td>
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<td>26</td>
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<td>F</td>
<td>71</td>
<td>20</td>
<td>25</td>
<td>32.8</td>
<td>N</td>
</tr>
</tbody>
</table>

Table 3.1:  
The Control population glycosylation data.  
(* refers to those patients treated with non steroidal anti-inflammatory drugs)
Table 3.2.
The mean GTase activity (cpm/mg protein.10^5±SEM) of the control population used in this study. OA=osteoarthritis. AS=Ankylosing spondylitis.

b. GTase and age.
Simple and polynomial regression analysis of the relationship between GTase activity and age within the sample population used in these investigations, indicated that there was no association between GTase activity and age.

c. Comparison of peripheral and tonsillar B cell GTase activity.
Lymphocytes were isolated from fresh tonsil tissue from 9 individuals (mean age 11 years, range 3-26 years) who had undergone tonsillectomy for benign tonsillar hypertrophy. FACS analysis has demonstrated that tonsillar lymphocytes are generally composed of greater than 65% B cells, and cell enrichment was therefore not attempted.

d. Results.
No difference in GTase activity was observed between the tonsil lymphocytic GTase activity and the control population (31 ± 6 vs 34 ± 4 cpm/mg protein.10^5; tonsil and peripheral B cell respectively).
iii. IgG glycosylation of the control population (Figure 3c).

Figure 3b.
Linear regression analysis of control (squares & straight line) and RA (circles & broken line) B cell and T cell GTase activity (mean cpm/mg protein $10^{-5}$). Pearson correlation coefficient $r = 0.770 \& 0.691$, Control & RA respectively; $p<0.001$.

Figure 3c.
The percentage of IgG lacking galactose ($G(0)\%$) in the control population.
All individuals, apart from 3, fell within 2 standard deviations of the mean of a quadratic regression curve relating serum IgG glycosylation (G(0)%) with age in a normal population (Figure 3c, Reference 122). These patients came from each of the 2 control groups, normal (n=1) and OA/AS (n=2), and one of the individuals with AS was taking NSAIDs.

There was no difference in mean G(O)% values between the 2 control groups (Normal, G(O)% 32 ± 3 and OA/AS G(O)% 36 ± 4). Regression analysis revealed a linear relationship between B/T cell GTase and G(0)% (Pearson correlation coefficient 0.345 & 0.347 respectively; p<0.05. Figure 3d).

Figure 3d.
Linear regression analysis of GTase activity (B cell = open squares & broken line, T cell = closed squares & straight line) and serum IgG(G0)%.
Pearson correlation coefficient r = 0.345 & 0.347 B & T cells respectively; p<0.05.
B. Patients with Rheumatoid Arthritis (Table 3.3).

i. Population description

32 patients, satisfying the revised ARA criteria for the diagnosis of Rheumatoid Arthritis (184), were selected at random from those attending the outpatient clinic. Their mean age was 59.9 years (range 28-88 years), and there were 27 females.

ii. IgG galactosylation of the RA population (Figure 3e).

The mean G(0)% of the RA population was significantly higher than the control population (39±3 v 32±3 cpm/mg protein.10^5, p<0.02. RA & control respectively).

iii. Lymphocytic GTase activity

(Figures 3f, g & h Tables 3.4 & 5)

a. GTase activity of the total population (Table 3.4).

Both B and T cell GTase activities were reduced by 45% and 29% respectively when patients were compared to the age-matched control population.
<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Sex</th>
<th>Age</th>
<th>ESR</th>
<th>Drugs</th>
<th>B cell GTase</th>
<th>T cell GTase</th>
<th>Disease activity</th>
<th>G(0)%</th>
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<td>108</td>
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Table 3.3:
The total rheumatoid arthritis patient population data.
Regression analysis revealed a linear relationship between the B and T cell GTase values (Pearson correlation coefficient $r = 0.691$, $p<0.001$. Figure 3b).

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<th>B CELL</th>
<th>T CELL</th>
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<td>CONTROL</td>
<td>34 ± 4</td>
<td>31 ± 4</td>
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<td>n = 32</td>
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<tr>
<td>RA</td>
<td>21 ± 3 *</td>
<td>22 ± 4 **</td>
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<td>n = 32</td>
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Table 3.4.
Galactosyltransferase activity (cpm/mg protein.10$^5$) in control and RA patients. *$p<0.01$. **$p<0.05$.

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<tr>
<td></td>
<td>NSAID only</td>
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<td>2</td>
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<td>(n=8)</td>
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</tr>
<tr>
<td></td>
<td>SASP &amp; another second line agent</td>
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<tr>
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<td>(n=15)</td>
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Table 3.5.
The pharmacological treatment used in the rheumatoid arthritis population.
b. GTase activity in relation to drug therapy (Reference 203).

Patients were divided into three groups, according to treatment (Table 3.5):

Group 1. No medication or non steroidal anti-inflammatory drugs (NSAIDs).

Group 2. Sulphasalazine (SASP) alone.

Group 3. Other second line or third line drugs.

All patients had been established on treatment for a minimum of two months.
Four of the nine patients in group 1, five of the eight patients in group 2 and
five of the 15 patients in group 3 were also taking NSAIDs.

c. B-Cell population (Figure 3f).

In groups 1 and 3, GTase was reduced by 60% (14.0 ± 5, mean cpm/mg
protein. 10^5 ± SEM; p<0.02) and 46% (19 ± 4 mean cpm/mg protein. 10^5 ±
SEM; p<0.02), respectively when compared to the total control population (35
± 4 mean cpm/mg protein. 10^5).

There was no difference when GTase activity of group 2 (SASP only) was
compared to the total control population (35±9 v 35±4 mean cpm/mg
protein. 10^5 ± SEM, group 2 and control respectively), but groups 1 and 3 were
significantly reduced when compared to group 2 (p<0.05).

Figure 3.f.
The effect of treatment on B cell GTase activity in patients with rheumatoid arthritis. In all RA patients, except those taking SASP, there is
a significant reduction in B cell GTase activity when compared to the
control population. *p<0.01  **p<0.02.
Regression analysis revealed a positive linear relationship between B/T cell GTase activity and G(0)% in group 2 patients, which did not reach statistical significance (Pearson correlation coefficient r = 0.504, p=0.09) and a negative linear relationship between B/T cell GTase activity and G(0)% in group 3 patients (Pearson correlation coefficient r = 0.490, p<0.05. Figure 3h).

d. T-cell population (Figure 3g).
In groups 1 and 3, GTase activity was reduced by 42% (18±4 mean cpm/mg protein.10^5 ± SEM, p<0.02), and 45% (17±3, mean cpm/mg protein.10^5 ± SEM, p<0.05) when compared to the control population (31 ± 4 mean cpm/mg protein.10^5).
There was no difference in GTase activity when group 2 (35±12 mean cpm/mg protein.10^5 ± SEM) was compared to the control population, but groups 1 and 3 were significantly reduced when compared to group 2 (p<0.05). Regression analysis revealed a positive linear relationship between B/T cell GTase activity and G(0)% in group 2 patients (Pearson correlation coefficient r = 0.801, p<0.01) and a negative linear relationship between B/T cell GTase activity and G(0)% in group 3 patients (Pearson correlation coefficient r = 0.462, p<0.05. Figure 3h).

\[\text{Figure 3g.}\]
The effect of treatment on T cell GTase activity in patients with Rheumatoid Arthritis. *p<0.05. **p<0.02.
Figure 3h.
Linear regression analysis of GTase activity (B cell = open squares and T cell = closed squares) in group 3 patients with serum IgG(G0)%. Pearson correlation coefficient $r = B$ cell (plain line) 0.490. T cell (dashed line) 0.462. $p<0.05$.

e. Conclusions.
The finding in the pilot study that B cell GTase was significantly reduced in RA patients has been confirmed by increasing the sample population from 19 to 32 individuals. Furthermore, it is apparent that T cell GTase activity is also reduced when compared to an age matched population. These findings have since been verified by another group (204).
Analysis of the disease modifying drugs being taken by these patients was carried out, to address the possibility that reduction in enzyme activity may be secondary to drug interaction. These data would indicate that drug therapy itself does not seem to reduce GTase activity, as there is a significant reduction in GTase activity when group 1 RA patients are compared to the control population (44% and 10% taking NSAIDs respectively) and no difference when group 1 and 3 patients are compared with each other.
These data do, however, indicate that patients taking SASP are more likely to have a normal GTase activity than those on other medication, or none at all, and a more detailed analysis of those patients commencing SASP therapy may be productive.
Sulphasalazine (salicylazosulphapyridine, SASP) is an azo compound of sulphonamide sulphapyridine (SP) and 5-aminosalicylic acid (5-ASA), and is cleaved by bacteria in the large intestine into SP and 5-ASA (205). It was initially synthesised as a combined anti-bacterial and anti-inflammatory agent (206).

SASP is now an established second line anti-rheumatic agent, useful in the treatment of RA (207-220), reactive arthritis (ReA) (221) and ankylosing spondylitis (AS) (222-225) and there is evidence that it may also be useful in psoriatic arthritis (226). It can also be used in inflammatory bowel disease (227). It has been shown to be as efficacious as gold and D-penicillamine in the treatment of RA (207-213), with few side effects (227a), and may also slow the progression of radiological disease (228).

Whether the pharmacologically active component is the intact molecule or a breakdown product has been much debated (205,228-230) but it has been shown that intact SASP is required for inhibition of mitogen effects (205).

Intriguingly, rectally administered 5-ASA has been shown to have more pharmacological activity in ulcerative colitis than SP or SASP (230), whereas the same has been found for oral SP in RA (206,228). Serum concentrations of SASP in patients do not reach those levels required to inhibit mitogen stimulation of lymphocytes (205), but in vitro inhibition of IgG, IgM and IgM rheumatoid factor production is inhibited (206). However, the serum concentrations of SASP in RA are low and unrelated to clinical effects (231), and it may be that the intact molecule exerts its effects by local suppression of lymphoid tissue in the small intestine (205), which in turn would affect the lymphocyte traffic pathways of cells emerging from the gut associated lymphoid tissue (232). This would imply that the mode of action of SASP in RA, AS and ReA is similar. In support of the hypothesis that arthritis may be perpetuated by exposure to foreign antigen, increased serum levels of IgA, including those directed against Klebsiella, have been observed (233,234) in patients with AS.

C. Is drug therapy related to the disease activity? (Table 3.6).

To ensure that analysis of data in relation to drug therapy is not biased by unduly active or inactive patients appearing in one particular group, the disease activity of each patient in each group was analysed (201).
Table 3.6. The relationship between the drug therapy of patients with rheumatoid arthritis with the clinical score of disease activity. DA-1 = Inactive/mild, DA-2 = moderately active, DA-3 = severely active.

i. Results.
Disease activity is not equally represented in the 3 treatment groups, and there are proportionately more individuals with DA 2 and 3 in drug group 2 and conversely, more individuals with DA 1 in drug group 1.

ii. Conclusion.
These results would indicate that if GTase activity were related to DA, reduced GTase in drug groups 2 and 3 may be related to patient selection. As it turns out drug groups 1 and 3 have equally reduced GTase activity and there is no difference between group 2 and the control population. This would suggest that a disease effect is being detected in groups 1 and 3 which is being altered (by SASP) in group 2.

It is interesting that 5 patients taking disease modifying drugs had clinically inactive disease, which would imply either the drug is having its desired effect or the disease is inactive. This would suggest that in future analyses differentiation should be made between disease quiescence as part of the natural history of the disease (ie: normal immune system) and that due to drug modification (ie. suppression of an abnormal immune system).
D. Does disease activity reflect GTase activity?

i. Results.
The mean B cell GTase activities (14 ± 3, 25 ± 7 and 24 ± 6) and T cell GTase activities (17 ± 4, 22 ± 5 and 27 ± 10) for DA-1 to 3 respectively (mean cpm/mg protein ± SEM.10^5) were not significantly different from the control population.

A negative correlation was found between T cell GTase activity and ESR in group 3 patients (r= -0.45, p<0.05), but there was no correlation between G(0)% and ESR. Likewise, no relationship was observed between GTase or G(0)% and disease activity indices.

ii. Conclusion.
These data would imply that there is no change in GTase activity as clinical disease activity increases. This could be due to an insufficient patient sample in each subset or may highlight the fact that abnormal cellular physiology may be present in RA without discernable clinical nor serological features.
CHAPTER 4.

LONGITUDINAL LYMPHOCYTIC GALACTOSYLTRANSFERASE STUDY OF PATIENTS WITH RHEUMATOID ARTHRITIS.

Is there a relationship between GTase activity, the percentage of IgG oligosaccharide chains lacking galactose, G(0)%, and other parameters of disease activity?

1. Introduction.

Human lymphocytic GTase has a physiological half-life estimated at 20 hours (65) and that for IgG is variable and dependent upon total IgG concentration (235), but it is probably in the range of 20 days.

It may, therefore, be hypothesised, that if G(0)% directly relates to GTase activity in peripheral blood lymphocytes, the measured G(0)% at a given time would correspond to the average enzyme activity over the previous 3 weeks.

With this in mind, it maybe envisaged that the relationship between G(0)% and GTase may fall into 3 categories:

a. Fluctuating disease activity:
GTase activity may predate a change in G(0)% and clinical activity score.

b. Sustained disease activity:
Increased non-fluctuating levels of G(0)% and decreased GTase activity, may be observed.

c. Quiescent disease:
Normal glycosylation parameters may be seen, except when GTase activity falls prior to a disease flare.

2. Longitudinal data analysis.
A. Patients (Tables 4.1,4.2,4.3).
All patients were caucasian and had sero-positive erosive disease. Details of disease activity and drug therapy are tabulated.

14 patients were investigated over an 18 month period. 11 patients were seen on 3 or more occasions and 3 patients were investigated twice. A total of 52 investigations were carried out.
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<th>AGE</th>
<th>ESR</th>
<th>DRUG Gp.</th>
<th>B Cell GTase</th>
<th>T Cell GTase</th>
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**TABLE 4.1:**
Rheumatoid arthritis patient data.
Group a: Longitudinal study (*abnormal G(0)% value).
Active disease with fluctuating glycosylation parameters and no sulphasalazine treatment.
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<th>DRUG Gp.</th>
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Table 4.2:
Rheumatoid arthritis patient data
Group b: Lounditudinal study (*abnormal G(0)% value).
Active disease with fluctuating glycosylation parameters and sulphasalazine treatment.
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<th>B Cell GTase</th>
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Table 4.3:
Rheumatoid arthritis patient data.
Group c: Longitudinal study.
Inactive disease with disease modifying drug treatment.
B. Results (Reference 236).

i. Overview of data.
No patients were investigated with clinically inactive disease who were not being treated with disease modifying drugs.
On the basis of the results obtained, the patients were divided into three clinical groups:

1. **Group a. (Table 4.1).**
   Active disease with fluctuating glycosylation parameters, who were not treated with sulphasalazine at any time.
This group comprised of 2 patients (18 & 25) with fluctuations in GTase activity that appeared to have a temporal relationship with G(0)% and clinical activity.

2. **Group b. (Table 4.2).**
   Active disease with fluctuating glycosylation parameters, who were treated with sulphasalazine.
This group comprised of 7 patients (5, 6, 8, 11,13, 31 & 32) who had generally normal GTase activities and abnormal G(0)% and 1 patient (9) with abnormal GTase and normal G(0)% measurements.

3. **Group c. (Table 4.3).**
   Inactive disease, but treated with disease modifying drugs.
This group comprised of 3 patients (3, 14 & 24) with generally abnormal GTase and G(0)% measurements and 1 patient (19) with generally normal GTase and G(0)% measurements.

ii. Individual patient data.

**Group a.**
Active disease with fluctuating glycosylation parameters, who were not treated with sulphasalazine.

**PATIENT 18:**
Sex: Female. Age at time of study: 47 years. Disease duration: 15 years.
This patient was treated with D-Penicillamine and had active disease throughout the study. Three sets of data are available for analysis. When first seen the B cell GTase is reduced to 2 cpm/mg protein.10^5 and may correspond to the abnormal G(0)% of 44% at that time and 39% at month 7, assuming the GTase activity remained depressed between months 0 and 7. At month 7 the B cell
GTase became raised to within normal limits, which is reflected in a normal G(0)% when she is next seen 6 months later. It must be presumed that the B cell GTase levels remain normal prior to testing at month 13. The other parameters of disease activity (clinical scoring and ESR) do not, however, show similar fluctuations.

PATIENT 25:
Sex: Female. Age at time of study: 62 years. Disease duration: 20 years.
This patient's disease was moderately well controlled with D-Penicillamine throughout the period of the study. Four sets of data are available for analysis. G(0)% is within 2SD for a 61 year old at months 0 and 9, and above the upper limit of normal at months 8 and 12. These measurements fluctuate corresponding to prior changes in B cell GTase activity, ESR and the clinical state of the patient.
When first seen B cell GTase was low, 11 cpm/mg protein.10^5, and this may have been maintained over the following 8 months when G(0)% increases from 37% to 51%. At this time disease activity has also increased from 1 to 2 and the ESR increased from 10 to 31 mm in 1 hour. At month 8, the B cell GTase activity was normal at 47 cpm/mg protein.10^5 and 1 month later this corresponded to a reduction in G(0)% from 51% to 35%. At month 9, however, B cell GTase was reduced to 24 cpm/mg protein.10^5 and 3 months later the G(0)% was increased to 49%.

Group b:
Active disease with fluctuating glycosylation parameters, who were treated with sulphasalazine.

PATIENT 5:
Sex: Female. Age at time of study: 43 years. Disease duration: 5 years.
This patient had active disease when first seen, and was treated initially with SASP with the addition of D-Penicillamine at month 5. There was clinical improvement over the five month period of the study. Three sets of data are available for analysis. G(0)% is abnormal initially and decreases from 52% to 31% over 5 months. GTase activity is normal initially, when SASP is being taken alone, and then
reduces to 2 cpm/mg protein by month five, which could indicate an impending disease flare.

PATIENT 6:
Sex: Female. Age at time of study: 74 years. Disease duration: 15 years.
This 74 year old female had active disease throughout the study and was successfully treated with SASP. Initially, the G(0)% was within 2SD of the mean, but at month 3 had risen to 54%, but fell to within the normal range at month 11. GTase activities were abnormal throughout.

PATIENT 8:
Sex: Female. Age at time of study: 34 years.
Disease duration: 4 years.
This patient had active disease when first seen, which was controlled with SASP. On the 2 occasions she was investigated, G(0)% measurements were raised, but B cell GTase activity was normal.

PATIENT 9:
Sex: Female. Age at time of study: 65 years.
Disease duration: 15 years.
This patient had active disease when first seen, which was controlled with SASP and the ESR seen to drop. However, G(0)% measurements were normal on the 2 occasions and the GTase activities were both abnormal. These paradoxical results cannot readily be explained in the context of either the patient's disease or treatment.

PATIENT 11:
Sex: Female. Age at time of study: 73 years.
Disease duration: 15 years.
This patient had adequately controlled disease with NSAID treatment for the first 9 months of the study, but her disease then became more active and she was treated with sulphasalazine. Her disease activity improved over the subsequent 3 months, but she was unable to tolerate the drug and it was stopped.

Five sets of data are available for analysis. Over the 12 month period of the study, there was normal GTase activity for the first 8 months, and decreased activity when her disease deteriorated. The G(0)% values are normal throughout
the study, in that they fall within 2 standard deviations (2SD) of the mean of a
ccontrol population for that age (Figure 3e). However, the G(0)% values
fluctuate and if glycosylation parameters over the last 4 months are analysed, a
staggered relationship between GTase and G(0)% may be seen. At month 8 the
B cell GTase is normal at 44 cpm/mg protein.10^5, which corresponds to a
G(0)% of 27% 1 month later. At month 9 the B cell GTase has decreased to 6
cpm/mg protein.10^5, and corresponds to a raised G(0)% at month 12 of 37%.
No such relationship, however, is found between months 0 and 1 and
commencng SASP (month 9) does not have an immediate effect on the already
low GTase values.

PATIENT 13:
Sex: Female. Age at time of study: 57 years.
Disease duration: 15 years.
This patient had active disease throughout the study. She was treated with D-
Penicillamine initially and SASP added later. There was a trend of reducing
GTase activity over the period of the study, which is paralleled by increasing
ESR. G(0)%, is however, abnormally raised for the first 8 months, but then
reduced to within normal limits. The G(0)% data is anomolous, as it does not
correspond with the GTase activities nor the clinical situation. It would be
important to record the effect SASP has on these parameters.

PATIENT 31:
Sex: Female. Age at time of study: 56 years.
Disease duration: 20 years.
This patient had increasing disease activity over the 15 month period of the
study, and was successfully treated with SASP and NSAIDs. Her disease
activity increased, at month 15 and GTase became reduced at this time also.
G(0)% was normal throughout the study.

PATIENT 32:
Sex: Female. Age at time of study: 70 years.
Disease duration: 8 years.
This patient had active RA throughout the study. She was treated with SASP
alone for the first 10 months, but D-Penicillamine was added at month 10. Four
pieces of data are available for analysis. Over 12 months the G(0)% reduced to
within the normal range and disease activity improved. When first seen B cell
GTase is 45 cpm/mg protein.10^5, which may have been maintained and is
reflected in a reduction in the G(0)% from 60% to 35% over 7 months. B cell GTase remains just below the lower limit of normal at months 7 and 12 and dipped to 4 cpm/mg protein at month 10, with no corresponding fluctuation in G(0)%.

**Group c: Inactive disease, but treated with disease modifying drugs.**

**PATIENTS 3, 14 AND 24:**
Sexes: Female. Age at time of study: 63, 59 & 64 years.
Disease duration: 15 years (patients 3 & 24).
10 years (patient 14).
These patients had clinically quiescent (63&59) and partially controlled disease throughout the study. Interestingly, their GTase activities were abnormal throughout the study, whereas the G(0)% values were within 2SD of the normal mean. The patients were treated with D-Penicillamine throughout, except patient 3 was only taking NSAIDs when first seen.

**PATIENT 19:**
Sex: Female. Age at time of study: 64 years.
Disease duration: 15 years.
This patient, treated with D Penicillamine, had clinically quiescent disease, with normal ESR and G(0)% measurements. B.GTase activities were low throughout, whereas T.GTase was low on 1 occasion (month 5).

**C. Conclusions**
Data from 14 patients has been analysed on 52 occasions, corresponding to outpatient visits over an 18 month period. The patients could be divided into 3 groups, depending upon disease activity and the use of sulphasalazine alone in treatment.

From these preliminary results some interesting data has been obtained.
Firstly, in patients with active RA and when SASP is not being taken (Patients 11[Early], 18, & 25) a temporal relationship between GTase activity, G(0)% and clinical activity can develop.
Secondly, in those with active RA and treated with SASP (patients 5, 8, 31, 32) GTase activity is generally within the normal range and may obscure any similar temporal relationship. In two patients studied before and after commencing SASP (11 & 13), no immediate effect on glycosylation parameters is noted.
These patients may not, however, be representative of those patients taking SASP as one could not tolerate the drug and the other was already taking D-penicillamine.

Thirdly, in those patients with inactive disease, and receiving second-line treatment other than SASP (patients 3, 14, 19, 24), GTase activity seems to be continually depressed, but G(0)% is not necessarily abnormal.

At this stage it may be concluded that:

1. A temporal relationship between glycosylation parameters and clinical activity is present in some patients with RA.

2. SASP tends to maintain GTase levels, but this need not result in changes in disease activity nor G(0)% levels.

3. Cellular dysfunction may still be present in a clinically inactive patient who is maintained on second-line anti-rheumatic agents, as shown by abnormal GTase activity.

The principal problems associated with this study are that the patients need to be observed more frequently and over a longer period of time, in the expectation that definite patterns will emerge.

However, there are a number of other confounding variables that are inherent in a study of glycosylation parameter changes. Firstly, GTase appears to be involved in normal cell physiology (75-78), and fluctuations in its level (both intracellular and cell surface) may be part of normal cell events. In this respect nothing is known about GTase and IgG-glycosylation fluctuations in the normal individual. Secondly, nothing is known about the control of GTase levels in the cell and thirdly, no kinetic data are available relating changes in intracellular GTase with G(0)%.

These data therefore only tell us that fluctuations of both GTase and G(0)% are occurring in patients with RA in certain patterns, which seem to be modified with drugs. It may be that in active RA GTase changes are predominantly related to the disease process, whereas they may not be in inactive disease. The real significance, if any, of this might be determined by studying a control group of normal individuals and RA patients more frequently and for a longer period of time.
CHAPTER 5.

IS THERE A SOLUBLE INTRACELLULAR INHIBITOR OF LYMPHOCYTIC GALACTOSYLTRANSFERASE IN B CELLS FROM PATIENTS WITH RHEUMATOID ARTHRITIS AND ARE THERE SELECTIVE ISOENZYME DEFECTS?

1. GTase inhibition.

A. Introduction:
Evidence has been presented to show that GTase activity is reduced primarily in the B cells of patients with RA. This may be:
   i. as a primary factor in pathogenesis,
   ii. as a secondary consequence of the rheumatoid inflammatory process.
Furthermore, reduced GTase activity may be due to a quantitative reduction of the enzyme within the cell or due to the presence of inactive enzyme. In patients with RA there may therefore be:
   i. an inherited defect in the gene encoding the enzyme.
   ii. an inhibitor of the enzyme, which may potentially exert its influence at a number of different sites, namely at the genetic level by inhibition of DNA translation and splicing or mRNA translation, or at the protein level by interacting with the enzyme itself.
To test whether an inhibitor - enzyme protein interaction was occurring within RA B cells, enzyme mixing experiments were carried out to determine whether enzyme from RA B cells could inhibit the catalytic activity of control B cell enzyme, or human milk GTase (HM Gtase).
Experiments were also performed to determine whether any differences observed with ovalbumin as a galactose acceptor, were also present with N-acetylglucosamine as an acceptor.

B. Methods.
i. Experiment 1: Mixing RA and control GTase.
Lymphocytic GTase was prepared, as previously described (chapter 2, P.65), from 10 patients with RA and six control individuals. Of the 10 RA patients, seven have previously been studied in other experiments, table 3.3, and only one of these had a normal B cell GTase activity on previously testing the sample. The three remaining RA patients had normal B cell GTase activities, on
previously testing the sample, and were being treated with SASP (Nos. 86 & 152) and a NSAID (140).

All RA patients, except 1 (152) had G(0)% greater than 2SD of the mean for the age.

There were fewer patients with severe disease (disease activity group 1: five patients (used in six assays), disease activity group 2: four patients (used in five assays), disease activity group 3: one patient (used in two assays).

Of the control individuals, three had no arthritis and the remaining three had OA (2) and AS.

B cell GTase activity (mean cpm of duplicates) was determined over one hour, as previously described, individually and then in the mixed patient and control preparations. The expected GTase activities were then compared with those actually obtained, and the additive, synergistic or inhibitory effects determined. Comparison between the actual/expected cpm difference (%), disease activity and G(0)% was then made.

ii. Experiment 2: Mixing patients' lymphocytic enzyme with Human milk GTase (HM.GTase).

a. Using N-Acetylglucosamine as the galactose acceptor.

Lymphocytic GTase was prepared, as previously described (chapter 2, page 65), from three patients with RA, two with disease activity 1, being treated with SASP, and one with disease activity 3, being treated with gold. The three control individuals had no arthritic disease.

B and T cell enzyme activity (mean cpm of duplicates) estimation was determined using a Dowex column, as previously described (chapter 2, P.72), to separate the bound from unbound acceptor molecules.

GTase was obtained from three patients with RA, who were known to have raised G(0)% and decreased GTase values, and three healthy controls, following the protocol as previously described. GTase activity was determined for the individual preparations and then when mixed with 50μU of HM.GTase.

b. Using ovalbumin as the galactose acceptor.

B cell GTase was prepared, as previously described (chapter 2), from three patients with RA, one with disease activity 1, being treated with a NSAID, and two with disease activity 2, being treated individually with SASP and D-Penicillamine.
B cell enzyme activity (mean cpm of duplicates) was determined, as previously described (chapter 2, P.65), using the high molecular weight acceptor ovalbumin, in three RA patients and three normal individuals separately and then when mixed with 50µU of HM.GTase.

C. Results (Reference 237).

i. Experiment 1 (Table 5.1).
There was no statistically significant difference between the actual and expected GTase activities when the 13 observations are analysed as a whole. The mean actual/estimated cpm difference for each member of the RA population tested was +2.2%, with a 95% confidence interval of 15.2, which is compatible with zero.
Appreciable inhibition was noted on one occasion (Patient 144), but synergism was noted in two RA patients (Nos. 152 and 173), with two similar observations occurring with patient 173 with different non-arthritis controls. No association is apparent between these four observations and disease activity, GTase activity and treatment. Likewise there was no difference in the mean actual/estimated cpm difference between the normal and arthritic control populations and there was no correlation between disease activity and the extent of inhibition, if observed. The two patients with group 3 disease activity had an increased actual GTase activity by 9% and 2% of that expected.
Of the three RA patients tested on two occasions with different controls (173, 182 & 184) there is consistency in the mean actual/estimated cpm difference in results obtained.

ii. Experiment 2A (Table 5.2).
The expected cpm for 50 µU HM.GTase in this experiment was 50.10^.
In all the assays performed, no inhibition of HM.GTase was detected with addition of either the RA or the control L.GTase. Nor were differences observed between the B and T cell preparations. Interestingly, a large degree of synergism was observed in each case.

iii. Experiment 2B (Table 5.3).
The cpm obtained in this experiment for 50µU HM.GTase was 20.10^.
Complete inhibition of H.GTase was not observed in any of the assays performed. However, in both the control and RA assays the mixed GTase activity was less than that expected, but there was not statistical difference
between the two groups. Unlike the assays performed with GlcNAc acceptor, no synergism was noted on mixing lymphocytic and human milk GTase.

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Table 5.1.
Experiment 1. A series of assays testing for inhibition of control B cell GTase by the addition of B lymphocytic GTase for patients with rheumatoid arthritis. Enzyme activity is expressed as mean cpm of duplicates. The control patients are divided into those with osteoarthritis (OA) and ankylosing spondylitis (AS) and the patient number is in parentheses in column RA.
### Table 5.2.
The effects of mixing B & T lymphocytic GTase with human milk GTase (50µU), equivalent to 50.10^3 cpm, when N-acetylglucosamine is used as an acceptor. Lymphocytic GTase activity is expressed as cpm.10^3.

<table>
<thead>
<tr>
<th>PATIENT SOURCE</th>
<th>CELL TYPE</th>
<th>BACKGROUND Activity</th>
<th>L.GTase Activity (corrected)</th>
<th>MIXED GTase Activity (corrected)</th>
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</thead>
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<td>RA</td>
<td>T</td>
<td>17</td>
<td>15</td>
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<tr>
<td></td>
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<td>483</td>
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<tr>
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<td>B</td>
<td>12</td>
<td>11</td>
<td>350</td>
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<tr>
<td>RA</td>
<td>T</td>
<td>15</td>
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<td></td>
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<td></td>
<td>B</td>
<td>13</td>
<td>14</td>
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### Table 5.3.
The effects of mixing B lymphocytic GTase from RA and control individuals with human milk GTase (50µU), equivalent to 20.10^3 cpm, using ovalbumin as an acceptor. B cell GTase activity is expressed as mean cpm.10^3 of duplicates.

<table>
<thead>
<tr>
<th>PATIENT SOURCE</th>
<th>BACKGROUND CPM</th>
<th>B CELL GTase</th>
<th>MIXED GTase (corrected)</th>
<th>% of EXPECTED CPM</th>
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<td>93</td>
<td>86</td>
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<td>CONTROL</td>
<td>8</td>
<td>119</td>
<td>136</td>
<td>98</td>
</tr>
</tbody>
</table>
D. Conclusions.
In experiment 1, the RA population mean cpm actual/estimated difference (%) was +2.2%. More than 50% of these patients had active disease (7 out of 13), and all but one had high G(0)% values. If a GTase intracellular inhibitor were present it should have been detected in this group of patients. Synergism greater than 20% was observed in three observation (two patients) and inhibition greater than 20% in one patient.
It is unlikely that variation in experimental method can explain these finding, as the coefficient of variation of the assay is within acceptable limits (4.8%), however, the combined endogenous (background) activities have been assumed to be equal to the sum of the individual endogenous (background) activities, which may not necessarily be the case, but would not result in a large degree of error.
In experiments 2 A & B, again no evidence of an intracellular inhibitor in the crude RA enzyme extract was obtained. Significant synergism was noted on mixing HM.GTase and GTase (from any source) when a Dowex column separation method was used with GlcNAc as the acceptor molecule. This was not observed when ovalbumin, a high molecular weight acceptor, was used. This may suggest that either there are different enzyme kinetics involved with the use of high and low molecular weight acceptors and that a co-factor may be present in lymphocyte extract that accelerates the reaction with GlcNAc, or that there are low molecular weight endogenous acceptors in the cell extract that can take part in the reaction and are separated on the Dowex column.
In conclusion, using three different experimental techniques, no evidence has been generated to suggest the presence of a soluble intracellular inhibitor of GTase within the RA lymphocyte.

2. GTase isoenzyme enzyme analysis.
Is reduction in B-cell GTase activity due to specific isoenzyme dysfunction rather than a generalised decrease in enzyme activity?

A. Introduction.
It is thought that different GTase isoenzymes are required for the transfer of galactose to different acceptors (57). On this assumption, potential variation in isoenzyme activity may be looked for by the use of different galactose acceptors in the catalytic reaction.
Methods

i. Preparation of acceptors.
Fetuin (from fetal calf serum), asialomucin (from bovine submaxillary gland) and N-acetylglucosamine (Sigma) were used in these experiments. Terminal sialic acid of fetuin was removed by mild acid hydrolysis and terminal galactose was removed from asialomucin and asialofetuin by periodate oxidation, sodium borohydride reduction and mild acid hydrolysis (238) as follows:
1. Mild acid hydrolysis of fetuin to remove sialic acid.
The acceptor was heated to 80°C for one hour in sufficient 0.05M sulphuric acid to allow it to dissolve. It was then neutralised with 1M sodium hydroxide and dialysed overnight against distilled water.
2. Periodate oxidation of the terminal non-reducing sugar.
Equal amounts of the acceptor and 0.02M sodium periodate in 0.05M sodium acetate buffer (pH4) were mixed and left in the dark at 4°C overnight. The reaction was stopped with an amount of glycerol equal to 1/10 of the solution of oxidised acceptor. The glycoprotein was then dialysed against distilled water.
3. Sodium borohydride reduction of the oxidised carbohydrate polymer to a polyalcohol.
Equal amounts of dialysed glycoprotein and 0.03M sodium borohydride in 0.3M potassium tetraborate buffer (pH 8.0) were mixed and incubated for 13 hours at 4°C and the reaction stopped by adjusting the pH to 5.0 with 1.0M acetic acid. The samples were then dialysed against distilled water.
4. Mild acid hydrolysis to cleave the acetal bond of the terminal residue.
Sulphuric acid was added to the dialysate to give a concentration of 0.025M. This was heated for 1 hour, cooled, neutralised with 1M sodium hydroxide, dialysed against distilled and then lyophilised.

ii. GTase assay.
B cell GTase from patients with active RA, together with raised G(0)% and normal individuals were assayed, as previously described, using the following galactose acceptors:

**Ovalbumin (OVA):**
12 RA patients & nine controls

**Asialo-mucin (AS-M):**
five RA patients & five controls

**Asialoagalacto-mucin (ASAG-M):**
seven RA patients & five controls
Asialoagalacto-fetuin (ASAF-F):
10 RA patients & 12 controls
N-acetylglucosamine (GlcNAc):
11 RA patients & 12 controls.

C. Results (Tables 5.4 & 5.5).

<table>
<thead>
<tr>
<th>ENZYME SOURCE</th>
<th>OVALBUMIN</th>
<th>ASAG-MUCIN</th>
<th>AS-MUCIN</th>
<th>ASAG-FETUIN</th>
<th>GlcNAc</th>
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<td>49.0±9.7</td>
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<td>n = 7</td>
<td>n = 5</td>
<td>n = 10</td>
<td>n = 12</td>
</tr>
<tr>
<td>CONTROL</td>
<td>22.3±6.0</td>
<td>3.6±1.4</td>
<td>40.3±9.4</td>
<td>6.4±1.3</td>
<td>57.3±15.6</td>
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<td>n = 9</td>
<td>n = 5</td>
<td>n = 5</td>
<td>n = 12</td>
<td>n = 12</td>
</tr>
<tr>
<td>% DECREASE</td>
<td>44.8*</td>
<td>9.1</td>
<td>43.7*</td>
<td>26.6</td>
<td>14.5</td>
</tr>
</tbody>
</table>

Table 5.4.
The mean (±SEM) B cell GTase activity (mean cpm/mg protein.10^5 of duplicates) using different acceptors, with the % decrease in activity for patients with rheumatoid arthritis.*0.05<p<0.1.

The ability of B cell GTase, from a normal population, to catalyse the transfer of galactose to different acceptors varied appreciably, and ranged from 57.5 cpm/mg protein.10^5 when GlcNAc was used as an acceptor, to 3.6 cpm / mg protein.10^5 when ASAG-mucin was used. This hierarchy was unchanged in the RA population.

When each acceptor group is analysed collectively, significant differences within the ovalbumin and AS-mucin groups are apparent, with 45% and 44% reductions in GTase activity respectively in the RA populations when compared to the control groups. There are no differences within the other three groups.

To further investigate this finding, the individual data (different acceptors and GTase samples) were compared as ratios of that found with ovalbumin (acceptor/Ova) and the average of these ratios compared with the control population. In this way an increase in the average RA ratio, compared to the control population, would indicate that GTase activity is not similarly depressed with that particular acceptor.
<table>
<thead>
<tr>
<th>ENZYME SOURCE</th>
<th>GlcNAC</th>
<th>ASAG-MUCIN</th>
<th>AS-MUCIN</th>
<th>ASAG-FETUIN</th>
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</thead>
<tbody>
<tr>
<td>RA</td>
<td>20.2</td>
<td>1.0</td>
<td>1.3</td>
<td>1.5</td>
</tr>
<tr>
<td>CONTROL</td>
<td>6.1</td>
<td>0.5</td>
<td>1.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Table 5.5. The mean acceptor ovalbumin ratios of individual RA and control B cell GTase activities.

An increase in average ratios was observed in all groups, except for AS-mucin where there was parity. This would serve to reinforce the original observation that the GTase enzyme defect is only observed, when ovalbumin and AS-mucin are used as acceptors.

D. Conclusions.

i. The ability of different galactose acceptors to take part in the GTase catalysed transfer of galactose from UDP varies within a control population.

ii. A similar hierarchy of acceptor ability is observed within the RA population.

iii. Reduction in GTase activity is observed within the ovalbumin and AS-mucin groups, which is confirmed by analysis of individual activities and comparing their acceptor:ovalbumin ratios.

iv. These results would suggest that specific GTase isoenzymes are reduced in RA, and that there is not an overall reduction in GTase isoenzyme activities.

v. This hypothesis should now be further tested by isoelectric focusing GTase from various sources and observing differences in quantity and activity of each isoenzyme band.
CHAPTER 6.

LYMPHOCYTIC GALACTOSYLTRANSFERASE DATA FROM OTHER DISEASE GROUPS.

Figure 6.a. The percentage of IgG lacking galactose in the tuberculosis, Crohn's disease and SLE patient populations. One of the three patients with SLE and raised G(0)% had Sjögren's syndrome.

1. Is GTase reduced in patients with Mycobacterium tuberculosis infection?

A. Overall data (Reference 239).

i. Introduction.
In a study of 21 patients with untreated pulmonary tuberculosis (PTB) (115), it has been observed that G(0)% is reduced in a similar fashion to that seen in patients with RA. To determine whether this may involve a similar synthetic defect to that hypothesised in RA, a study was performed to determine GTase activity in patients with untreated and treated PTB infections.
ii. Patients and methods (Table 6.1).
Eight patients (mean age: 38 years, range: 27-64 years. 5 males) were selected at random over an eight week period, from new inpatient admissions at UCH and The Brompton Hospital. three patients were subsequently investigated a total of eight times. GTase activity was determined in duplicate as previously described (Chapter 2, page 65).
32 patients without rheumatic autoimmune disease nor infections were used as control subjects (mean age: 54.5 years, range: 32-78 years 22 females). No association between age and sex had been observed within this population (Chapter 3).

iii. Results (Figures 6b & c).
Six patients (group 1) were studied seven days or less after starting treatment (Nos.143, 145, 149, 151, 220, 285), and their B and T cell GTase activities were reduced by 26% & 24% respectively when compared to 32 control patients (26 ± 9 vs 35 ± 4 and 22 ± 8 vs 29 ± 4 respectively; mean cpm/mg protein. 10^5 ± SEM; p<0.05).
Data were obtained from four patients (group 2) once established on conventional triple antibiotic therapy (eight days treatment or more); two of whom had been seen prior to the start of treatment. Mean B cell GTase activity (34 ± 9 mean cpm/mg protein.10^5±SEM) was similar to the control level and the mean T cell GTase activity (42 ± 18 mean cpm/mg protein.10^5 ± SEM; p=0.15 ) was increased by 44% when compared to the control population.
Three patients were studied when treatment was started and one month later (151,143,145). In all three patients the B and T cell GTase activities increased with treatment, and the increase in B cell GTase activity was statistically significant (B cell GTase: 9±7 vs 48±2 mean cpm/mg protein. 10^5 ± SEM; p<0.03. T cell GTase: 10±6 vs 28±7 mean cpm/mg protein. 10^5 ± SEM; p=0.08).

iv. Conclusions.
Eight patients were studied, generating 12 sets of enzyme activity data, and their GTase activities were reduced prior to treatment, when compared to a control population. In those patients who had received treatment, there was no difference in GTase activity in comparison to the control population. Furthermore, in three patients seen before and after treatment, there was a significant rise in B cell GTase activity with treatment.
B cell GTase activity (mean cpm/mg protein. $10^5$ of duplicates) in control individuals and those with active pulmonary tuberculosis. Group 1 are those patients who had treatment for 7 days or less and group 2 had treatment for 8 days or more.

T cell GTase activity (mean cpm/mg protein. $10^5$ of duplicates) in control individuals and those with active pulmonary tuberculosis. Group 1 are those patients who had treatment for 7 days or less and group 2 had treatment for 8 days or more.

B. Patient follow-up data (Table 6.1).

i. Introduction.

Three patients (a,b,&c) were seen on consecutive occasions following initial investigation as an inpatient. Clinically patients a and b presented with pyrexia, general malaise, a cough productive of sputum and changes on chest radiographs. Patient c presented with an intermittent fever and changes on chest
radiographs. In the first two patients, treatment was commenced empirically and later confirmed on sputum culture, whereas treatment in the last patient was started only after positive sputum cultures were obtained.

**ii. Clinical and experimental details.**

**a. Patient I.M. Age. 46 years. Male. (Nos. 143, 148 & 168)**

This patient was a vagrant and was seen prior to starting antibiotic therapy, 20 days after commencement of treatment, just before self discharge from hospital, and then again after two months off treatment, following readmission due to a relapse of symptoms. Unfortunately, subsequent to the last investigation the patient self-discharged and failed to re-present.

Over this period glycosylation parameters varied as follows: G(0)% were abnormal throughout and decreased from 60% to 54% following treatment (20 days) and increased to 59% after two months without treatment. B and T cell GTase increased with treatment (20 days) from 3 - 46 and 4 - 15 mean cpm/mg protein.10^5 respectively, and decreased to 4 and 0 mean cpm/mg protein.10^5 respectively on cessation of treatment.

**Conclusion.**

Both GTase and G(0)% vary inversely with the presence of active mycobacterial infection.

**b. Patient MS. Age. 64 years. Female. (Nos. 145, 250 & 417).**

This patient was seen on three occasions: on starting treatment, 6 months later whilst on antibiotic therapy and five months subsequent to this when the patient had been taking an additional 10mg of prednisolone for two months. Steroids were prescribed to modify active pulmonary inflammation.

Over this period glycosylation parameters varied as follows: G(0)% values were 60% at six months (abnormal), reducing to 39% (normal) at 11 months when steroids were commenced (no initial value is available). B and T cell GTase activity increased from pretreatment values of 2 & 5 mean cpm/mg protein.10^5 to 53 & 39 mean cpm/mg protein.10^5 respectively. Once treatment had been stopped and prednisolone started, these values reduced to 1 and 3 mean cpm/mg protein.10^5 respectively.

**Conclusions:**

GTase activity returned to normal levels with PTB treatment, and G(0)% decreased with this change in enzyme activity to normal. The fact that the G(0)% was elevated after six months treatment may be indicative of continuing
disease activity or inadequate therapy, but this level may have been less than the pretreatment level. Once treatment was stopped and prednisolone started reduced levels of GTase activity were recorded. This is unlikely to be due to the steroid treatment as drug effects on GTase activity have not been observed previously, although only studied in patients with autoimmune disease. The possibility may be that the patient is experiencing a recurrence of her infection, perhaps as a result of steroid treatment.

This patient was first investigated after 10 days of triple antibiotic therapy. Treatment was uneventful, and the patient was reinvestigated after 6 months therapy, when prednisolone was being taken. The patient subsequently moved house and was cared for elsewhere.
Over this period glycosylation parameters varied as follows: The initial G(0)% (at 10 days) was normal for his age at 33.3% and changed little over 6 months to 35.5%. B and T cell GTase activity increased from 22 to 46 and 21 to 30 cpm/mg protein.10^5, respectively.

Conclusions.
These results are in keeping with an improving clinical situation, although pretreatment data are not available and subsequent follow up has not been possible. Prednisolone treatment had no effect on these parameters.

C. Overall conclusions.
These data show that in five/six patients studied with PTB, GTase is reduced if estimated within seven days of starting antibiotic treatment, and in all three cases followed up GTase activity returned to normal after treatment.
Also, all of the patients had raised G(0)% when initially investigated, and normal values were observed in those established on antibiotic therapy.
These data suggest that GTase activity returns to normal rapidly, certainly at 20 days. The recovery time for G(0)% is difficult to assess as one patient received inadequate treatment and a pretreatment value is not available for the other.
<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Sex</th>
<th>Age</th>
<th>Group</th>
<th>B cell GTase</th>
<th>T cell GTase</th>
<th>G(0)%</th>
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</thead>
<tbody>
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<td>Carmichael</td>
<td>M</td>
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<td>28</td>
<td>1</td>
<td>23</td>
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<td>32</td>
</tr>
</tbody>
</table>

**TABLE 6.1:**
Pulmonary tuberculosis patient data.
Treatment group 1 = 7 days or less. Group 2 = 8 days or more.
Group 0 = 3rd observation, not used in statistical analysis.
2. Is GTase reduced in Crohn's disease?

A. Overall data (Reference 239).

i. Introduction.
Crohn's disease (CD) is one of the diseases so far identified as having raised levels of G(0)% (122). A preliminary study was therefore instituted to determine whether CD had similar GTase changes as found in RA.

ii. Patients and methods (Table 6.2).
The first 12 patients with a definite diagnosis of CD to attend the gastroenterology outpatient department at UCH in one month were studied. Four patients were followed up on one subsequent occasion.
Venesection, lymphocyte gradient separation, B and T cell enrichment and GTase activity estimation were carried out, in duplicate, as previously described (chapter 2). On the basis of clinical (patient's general symptoms, number of bowel motions, presence of blood and mucous in the motions etc.) and laboratory parameters (haemoglobin level, ESR, electrolytes, liver function, albumin etc), the gastroenterology team (Dr M Sarner) designated three patients as having "active" disease and the remaining nine patients as having "inactive" disease.

iii. Results (Figure 6.d).
In this study, there was no significant reduction in B and T cell GTase activity in those patients with inactive disease when compared to 32 control patients (32 ± 5 & 27 ± 5 vs 35 ± 4 & 29 ± 4) respectively; mean cpm/mg protein.10^5 ± SEM). However, a significant reduction in GTase activity was observed in the enzyme activities of those patients with active disease (11 ± 6 & 7 ± 6: mean cpm/mg protein.10^5 ± SEM; p <0.05 & <0.025, B and T cells respectively ).
Figure 6.d.
Comparison of GTase activity (mean cpm/mg protein $10^5$ of duplicates) in isolated T and B cell preparations in active and inactive Crohn's disease, from the total number of samples.

* $0.0125 < p < 0.025$. ** $0.025 < p < 0.05$.

iv. Conclusions.
In 12 patients studied, GTase activity is reduced in both the B and T cells in active disease, but not in inactive disease. To confirm these finding further a larger population needs to be sampled and compared to a control population of patients with other inflammatory bowel diseases, such as ulcerative colitis.

B. Follow up data on individual patients.

i. PATIENT RP, Age 44 years, Male. (Nos. 251 & 274).
This patient was seen on two occasions within four weeks. He was classified as inactive on each occasions and was taking NSAID for peripheral arthritis. Over this period his glycosylation parameters varied as follows: G(0)% 50 - 45 (abnormal); mean B cell GTase activity 42 - 12 cpm/mg protein $10^5$; mean T cell GTase activity 25 - 8 cpm/mg protein $10^5$.

Conclusion.
There was no change in clinical status over this period and the G(0)% was greater than the 2SD range for his age on both occasions. The drop in both B
and T cell GTase activity could indicate a relapse in disease. Further studies will be interesting, especially in view of the patient’s arthritic symptoms.

ii. PATIENT HS, Age: 38 years, Male. (Nos. 229 & 425).
This patient was seen on two occasions with seven months separating each study. On each occasion disease was assessed as active, and the patient was treated with azathioprine and prednisolone.
Over this period his glycosylation parameters varied as follows: G(0)% 45 - 38 (abnormal); mean B cell GTase activity 25 - 1 cpm/mg protein.10^5; mean T cell GTase activity 24 - 1 cpm/mg protein.10^5.

Conclusion.
These data are similar to those found with patient RP, where G(0)% values were initially normal with normal GTase activity that decreased on subsequent investigation. This may again indicate a relapse in disease.

iii. PATIENT CN, Age: 65 years; Male. (Nos. 175 & 413).
This patient was seen on two occasions nine months apart. On the first occasion the patient had active disease, and was being treated with sulphasalazine and prednisolone; his ESR was 75 mm/hr. On the second occasion the patient was being treated with SASP only and his disease was inactive with an ESR of 10 mm/hr.
Over this period glycosylation parameters varied as follows: G(0)% 38.6(abnormal) - 20.0; mean B cell GTase activity 0 - 10 cpm/mg protein.10^5; mean T cell GTase activity 0 - 10 cpm/mg protein.10^5.

Conclusion.
These data would be consistent with the patient's disease improving, and it would be of interest to determine whether both B and T cell GTase activities increase further.

iv. PATIENT C.G; Age: 25 years; Female. (Nos. 171 & 394).
This patient was seen twice over a nine month period, during which her disease activity was initially considered active and successfully treated with azathioprine, SASP, and prednisolone. The patient was taking these drugs at the time of each study and ESR remained unchanaged at 5 mm/hr.
Over this period glycosylation parameters varied as follows: G(0)%: 42 (abnormal)- 20 ; mean B cell GTase activity 19 - 8 cpm/mg protein.10^5 and mean T cell GTase activity: 2 - 4 cpm/mg protein.10^5.
Conclusion.
The IgG glycosylation data are consistent with active disease being controlled. However, although G(0)% values return to within the normal range, GTase activities do not.

C. Overall conclusions.
A small study has been carried out to determine whether lymphocytic GTase activity is affected in CD. There is a suggestion that it is low in active disease but the data would indicate that the relationship between G(0)% and GTase activity is again not a simple one and may not vary synchronously with disease activity. Unlike the PTB study no clear cut improvement in GTase activity was observed with disease improvement. This could indicate continued subclinical activity of the Crohn's disease.
<table>
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<tr>
<th>No.</th>
<th>NAME</th>
<th>SEX</th>
<th>AGE</th>
<th>DRUGS</th>
<th>G(%)</th>
<th>ESR</th>
<th>B Cell GTase</th>
<th>T Cell GTase</th>
<th>ACTIVE</th>
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<td>170</td>
<td>Buss</td>
<td>F</td>
<td>40</td>
<td>Azathioprine</td>
<td>20.8</td>
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<td>Davda</td>
<td>F</td>
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<td>Prednisolone</td>
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<td>n/d</td>
<td>19</td>
<td>67</td>
<td>-</td>
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<tr>
<td>231</td>
<td>Dawe</td>
<td>M</td>
<td>46</td>
<td>SASP</td>
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<td>-</td>
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<td>24</td>
<td>-</td>
</tr>
<tr>
<td>171</td>
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<td>M</td>
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<td>M</td>
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<td>-</td>
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<td>Rathod</td>
<td>M</td>
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<td>18</td>
<td>-</td>
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<tr>
<td>219</td>
<td>Spito</td>
<td>M</td>
<td>38</td>
<td>NIL</td>
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<td>42</td>
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<td>229</td>
<td>Steinhof</td>
<td>M</td>
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<td>+</td>
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<td>Turner</td>
<td>F</td>
<td>25</td>
<td>NIL</td>
<td>40.4</td>
<td>n/d</td>
<td>28</td>
<td>29</td>
<td>-</td>
</tr>
</tbody>
</table>

**TABLE 6.2:**

Crohn's disease patient data.
(NSAID = non steroidal anti-inflammatory drug, SASP = sulphasalazine,
AZ-(SA)-Pred = Azathioprine + (sulphasalazine)+prednisolone)
3. Is GTase reduced in patients with systemic lupus erythematosus?

A. Overall data.

i. Introduction.

It has been previously reported that only those patients with SLE associated with Sjögren's syndrome have significantly raised G(0)% values (115). However, a smaller study, using quantitative carbohydrate analysis, has observed that IgG-Fc galactose is reduced in some patients with SLE alone (120), which has been the subject of discussion (240).

ii. Patient details & Methods (Table 6.3).

12 patients, satisfying the ARA criteria for SLE (185), were selected at random from those attending the SLE clinic. Both B and T cell GTase activities were estimated in duplicate, as previously described (chapter 2), and compared to a control age-matched population of individuals. Disease activity was assessed as inactive (1), mild (2), moderate (3) & severe (4) (241).

iii. Results (Figure 6e).

a. GTase activity (239).

Both mean B and T cell GTase activities were significantly reduced when compared to a control population (10 ± 3 (p<0.01) & 7 ± 2 (p<0.001) vs 27 ± 5 & 34 ± 6, B & T cells respectively: mean cpm/mg protein.10^5 ± SEM).

<table>
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<th>DISEASE ACTIVITY</th>
<th>1</th>
<th>2 - 4</th>
</tr>
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<tbody>
<tr>
<td>B CELL GTase</td>
<td>6 ± 1 *</td>
<td>7 ± 4 **</td>
</tr>
<tr>
<td>T CELL GTase</td>
<td>15 ± 5 §</td>
<td>7 ± 2 §§</td>
</tr>
</tbody>
</table>

Table 6.3.

The variation in B and T cell GTase activities (mean cpm/mg protein.10^5) with disease activity in patients with SLE.

* p<0.02. ** p<0.01. § p=0.05 (one tail). §§ p<0.01.
Figure 6e.
Comparison of GTase activities (mean cpm/mg protein.10^5 of duplicates) in isolated T and B cell preparations in age matched patients with systemic lupus erythematosus and normal individuals. *p<0.01. **p<0.001.

B. Disease activity (Table 6.3).
When these data are divided according to disease activity both groups have significantly decreased GTase activity when compared to the control population, although the reduction seen in group one T cell activity is not so pronounced as in the other categories.

C. Drug effects (Table 6.4).
The majority of patients (n=seven) were taking steroids. Six were taking prednisolone and one, who was pregnant, was taking dexamethasone, but being given this because of cerebral secondaries from her coincident carcinoma of the breast. Four patients were taking immunosuppressive medication, one was taking an antidepressant, and chloroquine phosphate was being taken by one patient.
Both the mean B and T cell GTase activities in those patients either on no medication (four patients: 10.2 ± 5 & 5.8 ± 2 mean cpm/mg protein.10^5; B (p<0.05) & T (p<0.05) respectively) or steroids (seven patients, 5.3 ± 1.1 & 12.0 ± 4.3 mean cpm/mg protein.10^5; B (p<0.005) & T (p<0.05) respectively) were significantly reduced when compared to the control population. The
patient taking chloroquine phosphate had similar GTase activities to the other two groups.

D. IgG glycosylation, ESR and disease activity (Table 6.4).

Of the 12 patients studied only three had abnormal $G(0)\%$ values. In two of these patients, 323 and 327, $G(0)\%$ was 48 & 54 respectively, which was associated with active disease, raised ESR (80 & 29 mm/hour respectively) and Sjögren's syndrome with arthritis in one (327). The third patient (340), had $G(0)\%$ of 37, and was pregnant, with inactive lupus and a raised ESR (63 mm/1 hour), but had coincident metastatic carcinoma of the breast. In three patients (315, 316 & 320) there was active disease (DA 2 & 4) associated with raised ESR and arthritis in the latter two, but normal $G(0)\%$, and in a further three patients (324, 338 & 339) there was normal ESR and $G(0)\%$, associated with arthritis in one (338). In the remaining three patients (314, 322 & 328) all parameters, apart from GTase activity, were normal and one patient (328) had arthritis.

E. Conclusions.

B and T cell GTase activities were reduced in those patients with SLE when compared to a normal population. This appears to be unrelated to disease activity, the medication the patient is taking (ie: prednisolone or chloroquine in this study) or the presence of arthritis.

It has been previously reported that $G(0)\%$ is generally only reduced in those patients with SLE and Sjögren's syndrome (115). Insufficient GTase data is available to determine whether these patients are distinct from other patients with SLE (ie: is their GTase activity reduced even further?), but it is evident from the $G(0)\%$ values of those patients studied that reduced $G(0)\%$ is not as common as that found in patients with RA. Of the three lupus patients with a raised $G(0)\%$, one had coincident Sjögren's syndrome.

Further data needs to be collected from those patients taking other medication (ie: Chloroquine) and from those with Sjögren's syndrome. Sequential analysis of glycosylation parameters from individual patients would also be useful in determining whether disease activity affects these glycosylation parameters.
<table>
<thead>
<tr>
<th>No.</th>
<th>NAME</th>
<th>SEX</th>
<th>AGE</th>
<th>DRUGS</th>
<th>DIAGNOSIS</th>
<th>G(0)%</th>
<th>ESR</th>
<th>B cell GTase</th>
<th>T cell GTase</th>
<th>DA</th>
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<tr>
<td>314</td>
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<td>22</td>
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<td>SLE/Nephritis</td>
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<tr>
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<td>F</td>
<td>47</td>
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<td>SLE/Sjogren's</td>
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<td>63</td>
<td>6</td>
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<td>Depixol</td>
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<td>SLE/Sjogren's</td>
<td>54*</td>
<td>29</td>
<td>3</td>
<td>2</td>
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<tr>
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<td>340</td>
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<td>F</td>
<td>27</td>
<td>Dexameth.</td>
<td>SLE 7/12 preg</td>
<td>37*</td>
<td>63</td>
<td>5</td>
<td>11</td>
<td>1</td>
</tr>
</tbody>
</table>

**TABLE 6.4:**
SLE Patient data (* abnormal G(0)% values).
4. Is GTase reduced in CD5 positive B cells from patients with chronic lymphocytic leukaemia?

A. Introduction.
It known that B lymphocytes bearing the CD5 surface marker are more common in the peripheral blood of patients with RA, when compared to a normal population (135-137). Although separation of CD5 positive B cells is possible from peripheral blood by sorting cells labelled with fluorescent anti-CD5 monoclonal antibodies insufficient cells would be obtained to enable GTase activity estimation using the present technique. However, cells with the CD5 surface marker are the predominant cell in chronic lymphocytic leukaemia (CLL) (242), and lymphocytes from these patients are available in sufficient quantity for GTase analysis.
This study was therefore designed to test whether there was any evidence to suggest that decreased GTase in RA B cells is due to an over representation of CD5 positive B cells in RA peripheral blood.

B. Patients and Methods.
Lymphocytes from 15 patients (mean age: 68.9, range 81 - 60 years, nine females), with a diagnosis of CLL, were obtained over a 3 month period by the methods previously described (chapter 2), and the predominance (>65%) of CD5 positive B cells confirmed by fluorescent antibody cell sorting. The diagnosis of CLL was made on clinical and haematological criteria. None of the patients was suffering a blast crisis, and all were otherwise well. Cytotoxic medication was not being taken by any of the patients. GTase activity determination was carried out, in duplicate, on the monocyte depleted lymphocyte population, as previously described (chapter 2).

C. Results (Figure 6f).
The mean GTase activity of the whole lymphocyte population in those patients with CLL was significantly reduced when compared with 15 age matched (mean age 67 years, range 57 - 78 years) normal individuals (36 ± 7 vs. 21 ± 3; cpm/mg protein.10^5 ± SEM. p< 0.05 ).
Figure 6f.
Comparison of B cell GTase activity (mean cpm/mg protein$\times 10^5$ of duplicates) in normal individuals and patients with chronic lymphocytic leukaemia. *p<0.05.

D. Conclusions.
That the GTase activity of a whole lymphocyte population, containing at least 65% B cell CD5 positive clones, is reduced when compared to that from control B cells is interesting for two reasons.
Firstly, serum and tissue GTase activity estimation in all malignancies measured by other workers to date has been raised and is thought to be useful as a tumour marker (200,243), and secondly, the association of an enzymatic abnormality with a class of B cells (CD5 positive) that may be responsible for the production of autoantibodies is intriguing and may be pertinent.
These data would indicate that it would now be important to determine GTase activity in CD5 positive B cells from patients with RA, and the reduction would be expected to be greater the better the B cell enrichment.
ARE THERE GTase ACTIVATOR GENE REARRANGEMENTS IN PATIENTS WITH RHEUMATOID ARTHRITIS?

1. Introduction.
It has been previously described (chapter 1, page 48) that G(0)% is increased in a restricted number of disease (RA, TB, Crohn's disease and SLE associated with Sjögren's syndrome), and that this defect is unlikely to be due to postsecretory degradation as β-galactosidase levels are not increased in RA and cultured B cells from RA patients secrete defective IgG in comparison to normal B cells.

GTase is found in the golgi and on the cell surface of normal lymphocytes (chapter 1, page 42), and participates in a wide range of biological activities. This study has reported a reduction in lymphocytic GTase activity in RA (chapter 3), which is unlikely to be due to the presence of an intracellular enzyme inhibitor (chapter 5), but may be due to different GTase isoenzyme activities (chapter 5).

Alternatively, reduced levels of GTase activity may be due to either alterations in the structure of the gene encoding the enzyme or, alternatively, to decreased levels of gene expression caused by regulatory defects.

A cDNA clone (λgt 2) for a GTase associated, calmodulin-regulated, activator gene (GTA gene) has been isolated from a human liver λgt 11 expression library, using a polyclonal antiserum to affinity purified bovine enzyme (244). It is thought that expression of this gene results in a protein kinase that phosphorylates GTase, and hence regulates its activity (Dr V Kidd).

The authenticity of this cDNA clone has been convincingly demonstrated (244) by:

a. Increased RNA production, detected with the cDNA probe, concomitant with increased GTase activity.

b. GTase production using eukaryotic expression vectors containing the λgt 2 insert.

The human cDNA clone was subsequently used to localize the gene for GTA to human chromosome 4 by Southern blot analysis of a somatic cell hybrid panel. In a recent study of patients with acute lymphoblastic leukaemia, restriction fragment length polymorphisms have been observed using a 1.6Kb cDNA probe, with Hind III and EcoR1 enzyme digestion of the DNA (Personal communication Dr V. Kidd).
Using GTA gene probes, alteration in GTA gene structure was investigated in RA patients to determine whether GTA rearrangements are responsible for the reduced GTase activities found in RA patients.


A. Lymphocyte preparation.
Peripheral blood was obtained by venesection into heparinized bottles, and the erythrocytes lysed with ammonium chloride lysis buffer for 15 minutes at 4°C. The leucocytes were washed twice in Hank's balanced salt solution. B and T cell enriched preparations were made as previously described (chapter 2).

B. GTA probes (Figure 7a).
The following probes for the GTA gene locus were used:

1. 1.6Kb 3' cDNA.
2. 1.5Kb 5' cDNA.
3. 2.0Kb 3' cDNA.

![Restriction enzyme map of the full length GTA cDNA.](image)

**Figure 7.a.**
Partial restriction enzyme map of the full length GTA cDNA. Thick bar denotes coding region. Restriction enzyme sites indicated as follows: P: Pst1, Bg: BglII, Pv: PvuII, B: BamH1, K: Kpn1, and E: EcoR1. The probes isolated from this cDNA are shown below the restriction map.

C. Patient details.
DNA from a total of 24 RA patients, with raised G(0)%, and 15 normal individuals were digested with EcoR1 and/or HindIII and other restriction endonucleases.
D. Southern blotting.

DNA was isolated from peripheral blood leucocytes by proteinase K digestion and phenol - chloroform extraction using standard techniques (245). 10μg DNA was digested with 25-50 units of restriction endonucleases in buffer for 16 hours at 37°C. Parallel digestion of 0.5μg bacteriophage λ DNA using a 3μl aliquot taken from the genomic DNA digest served as an indicator of completeness of digestion. Additional enzyme was added to any incompletely digested samples.

Restriction fragments were separated on agarose gels (0.5% - 1.0%), followed by partial acid hydrolysis in 0.125N HCl for 20 minutes, denaturation in 0.5N NaOH/1.5M NaCl for 40 minutes and neutralization using 1.5M NaCl /0.5M Tris pH 8.0 for 40 minutes. Gels were blotted overnight onto nylon membranes (Hybond-N, Amersham) followed by cross linking of DNA to the nylon by 5 minute UV irradiation.

Blots were pre-hybridized for a minimum of 2 hours at 65°C in 3X SSC, 0.1% SDS and 50mg/ml denatured salmon sperm DNA. Hybridization was carried out for 16 hours at 65°C in hybridization mixture ( pre-hybridization mixture containing 10% Dextran sulphate) together with the appropriate 32P-labelled cDNA probe. The probes were labelled using the oligo-labelling Multiprime system (Amersham) and denatured prior to use by heating to 95°C for 5 minutes. Non-specifically bound radioactivity was removed from membranes by washing down to a stringency of 0.3X SSC at 65°C. Autoradiography was carried out using screened X-ray cassettes at -70°C with Hyperfilm-MP (Amersham).

Following autoradiography, probes were removed from the Southern blots by incubation in 0.4N NaOH for 30 minutes at 45°C followed by a similar incubation in 0.1x SSC, 0.1% SDS and 0.2M Tris-HCl (pH 7.5). The blots were then re-probed with labelled cDNA corresponding to other regions of the GTA gene.

3. Results (Reference 237).

(Tables 7.1, 7.2, 7.3, Figures 7 b,c, d & e).

When EcoR1-digested DNA was hybridized with the 1.5Kb 5' probe, major bands of approximately 10.5, 7.4 and 2.5 Kb were seen in all patients and control individuals (Figure 7b). Faint bands of approximately 15.0Kb and 2.3Kb (latter not visible in this figure) were usually also present, and showed variability in intensity between individuals, which was not always reproducible, suggesting that they may represent cross-hybridizing related sequences.
When the 1.6Kb 3' probe was used to reprobe the EcoR1 blots, a single band of approximately 4.0Kb was seen in all individuals tested, whereas reprobing with the 2.0Kb 3' probe revealed this band together with weak hybridization to the 10.5Kb band detected with the 5' probe.

Using HindIII-digested DNA (Figure 7c), hybridization with the 1.5Kb 5' probe gave bands of approximately 17.0 and 5.1, whilst both the 2.0 Kb and 1.6 Kb 3' probes gave bands of approximately 5.1 Kb and 4.0 Kb. The Hind III patterns were similar in all individuals tested.

DNA from a total of 24 RA patients and 15 normal individuals were digested with EcoR1 and/or HindIII. In none of the patients analysed was there any evidence for gross structural changes in the GTase activator gene.

15 further restriction endonucleases were used to screen 8 normal individuals to identify any enzyme that might be informative with RA DNA.

The enzyme BgI II detected a polymorphism using the 1.5 Kb 5' GTA probe (figure 7d), and bands of approximately 6.5, 4.5, and 1.6 Kb were seen in all eight normal individuals. Polymorphism for 19Kb and 12 Kb bands was identified and homozygous and heterozygous individuals observed. One of these eight individuals (Lane 6) also reproducibly exhibited an additional band of approximately 7.8 Kb. This individual also showed a polymorphism with the enzyme Bam H1.

Analysis of DNA from 30 RA patients revealed similar findings using BglII (figure 7e), but no significant difference in the occurrence of these 2 allotypes was found when compared to the patterns of 34 normal controls. Two of these RA patients also possessed the additional 7.8 Kb band seen in the normal control. When BglII blots were re-probed with the 2.0 Kb 3' GTase they all showed the same banding pattern.

This lack of polymorphism seen with the probe for the 3' untranslated region, together with the reproducibility of these findings, suggests that the polymorphisms seen in the protein coding region of the gene are not due to incomplete restriction enzyme digestion.
Figure 7.b.
Southern blot of the EcoR1-digested DNA separated on a 0.5% agarose gel and hybridized with the 1.5 Kb 5' GTA probe. Molecular size marker is bacteriophage λDNA digested with HindIII. Lane 1 is DNA from a normal control, lanes 2-8 are DNA from RA patients. A very weak band of approximately 2.3 Kb is just discernable in most lanes on the original autoradiograph.
Figure 7.c.
Southern blot of HindIII digested DNA separated on a 0.5% agarose gel and hybridized with the 1.5 Kb 5' and 2.0 Kb 3' GTA probe.
Figure 7.d.
Southern blot of BglII digested DNA separated on a 0.5% agarose gel and hybridized with the 1.5 Kb 5' GTA probe. Lanes 1-8 are DNA from eight normal individuals.
Figure 7.e.
Southern blot of the BglII-digested DNA separated on a 0.5% agarose gel. Lanes 1-4 are DNA from RA patients hybridized with the 1.5Kb 5' GTA probe.
<table>
<thead>
<tr>
<th>cDNA PROBE</th>
<th>EcoR1-DIGESTED DNA</th>
<th>HINDIII-DIGESTED DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5Kb 5' GTA</td>
<td>(15.0)+10.5+7.4+2.5+(2.3)</td>
<td>17.0+5.1</td>
</tr>
<tr>
<td>2.0Kb 3' GTA</td>
<td>(10.5)+4.0</td>
<td>5.1+4.0</td>
</tr>
<tr>
<td>1.6Kb 3' GTA</td>
<td>4</td>
<td>5.1+4.0</td>
</tr>
</tbody>
</table>

TABLE 7.1: 
The band sizes (Kb) obtained from each of 24 patients and 13 normal individuals for EcoR1 digestions and from 18 patients with rheumatoid arthritis and from 15 normal individuals for HindIII digestions. Bands in ( ) are weak.

<table>
<thead>
<tr>
<th>BANDS</th>
<th>RHEUMATOID ARTHRITIS</th>
<th>CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>19Kb Homo</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>12Kb Homo</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>TOTAL</td>
<td>30</td>
<td>34</td>
</tr>
</tbody>
</table>

TABLE 7.2: 
Number of individuals homozygous or heterozygous for BglII polymorphism.
<table>
<thead>
<tr>
<th>ENZYMES</th>
<th>BAND SIZE (Kb)</th>
<th>POLYMORPHISMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PvuII</td>
<td>3.9+1.3+1.0+0.8+0.7+0.5</td>
<td>None detected</td>
</tr>
<tr>
<td>EcoR1</td>
<td>(15)+10.5+7.4+2.5+(2.3)</td>
<td>Variability in weak bands</td>
</tr>
<tr>
<td>BamH1</td>
<td>(23)+6.2+4.1+1.2</td>
<td>9.0Kb band in 1 individual</td>
</tr>
<tr>
<td>AvalII</td>
<td>6.5+1.7+1.4+1.2+1.1+1.0+0.5+0.3</td>
<td>None detected</td>
</tr>
<tr>
<td>BgIII</td>
<td>19 &amp;/or 12+6.5+4.5+1.6</td>
<td>19 &amp; 12 Kb homo &amp; heterozygous bands. 7.8Kb band in same individual as BamH1</td>
</tr>
<tr>
<td>HindIII</td>
<td>17+5.1</td>
<td>None detected</td>
</tr>
<tr>
<td>Msp1</td>
<td>8 small bands&lt;1.0</td>
<td>None detected</td>
</tr>
<tr>
<td>Nar1</td>
<td>50</td>
<td>None detected</td>
</tr>
<tr>
<td>NheI</td>
<td>25+6.0</td>
<td>None detected</td>
</tr>
<tr>
<td>PstI</td>
<td>(5.7)+(3.6)+2.8+2.3+1.5+0.8+0.5</td>
<td>None detected</td>
</tr>
<tr>
<td>SalI</td>
<td>&gt;50</td>
<td>None detected</td>
</tr>
<tr>
<td>Sau961</td>
<td>50+30+10.0+5.0</td>
<td>None detected</td>
</tr>
<tr>
<td>ScaI</td>
<td>30+20</td>
<td>None detected</td>
</tr>
<tr>
<td>SmaI</td>
<td>50+23+10+8.4+5.4+4.5</td>
<td>None detected</td>
</tr>
<tr>
<td>XhoI</td>
<td>50</td>
<td>None detected</td>
</tr>
</tbody>
</table>

**TABLE 7.3:**
Galactosyltransferase activator gene RFLPs in 8 normal individuals using a 1.5Kb 5' probe.
The data from EcoR1 and Hind III restriction enzyme digestion of RA DNA data and subsequent analysis with GTA gene probes, would suggest that gross structural changes in this gene are not responsible for the reduced levels of GTase seen in peripheral lymphocytes of patients with RA. This does not exclude the possibility of alterations in the gene encoding the GTase component itself, although demonstration that G(0)% fluctuates with disease activity (114) would argue against such a defect because normal galactosylation is restored during remission.

It would now be important to determine whether transcriptional regulation of GTase genes can occur with mediators of inflammation, for example cytokines (see discussion).

The RFLP's observed using the enzyme BglII indicate that the GTA gene exists as different polymorphic alleles, as revealed by homo- and heterozygosity for the 19Kb and /or 12 Kb bands, and it may be that subtle alterations of the GTA gene locus associated with specific RFLPs may contribute toward the RA phenotype. Multigeneration family studies will be required to clarify this.

It would now be appropriate to investigate the level of expression of the GTase activator gene in a RA population and also in those normal individuals with low GTase and raised G(0)% levels.
ARE THERE GTase CHANGES IN A MOUSE STRAIN THAT EXHIBITS SPONTANEOUS AUTOIMMUNITY AND ARTHRITIS?

1. Introduction.
The congenic MRL lpr/lpr and +/+ mice were initially proposed as a model for SLE (194), but they also have pathological similarities to RA (195,196) and Sjögren's syndrome (197). The CBA/Ca mouse strain, however, has no spontaneous autoimmune tendency and is used as a control strain in these studies.

Mouse IgG has been shown to contain complex N-linked biantennary sugar chains, which are almost totally fucosylated and contain a bisect GlcNAc (246). The demonstration that glycosylation changes (GTase activity and G(0)%
) occur prior to the development of arthritis in humans would be important and would imply that these events play a primary role in the pathogenesis of RA. The use of available animal models to study these changes may therefore help shed more light on disease mechanisms in RA.

An initial study to determine whether GTase changes are present in MRL B-cells was therefore carried out.

Two duplicate experiments, using pooled B cell preparations from peripheral blood and the spleen of both MRL lpr/lpr, MRL +/+ and CBA/Ca mice were carried out.

A. Peripheral B cell preparation:
Whole blood was obtained from 37 MRL lpr/lpr mice (average age 22 weeks; 18 male, 19 female), 37 MRL +/+ mice (average age 21 weeks; 30 male, 7 female) and 30 CBA/Ca mice (average age 23 weeks, 60 male). Lymphocytes were obtained by gradient centrifugation of whole blood, monocyte depleted by plastic adherence and T cell depleted using anti-Thy1 monoclonal antibody and complement.
B. Splenic B-cell preparation.
Spleens were taken from the same mice, cell suspensions prepared, red cells lysed with ammonium chloride, and then monocyte and T cell depleted as described.

C. GTase enzyme assay.
The resulting B cell enriched preparations were resuspended in 100 mM Tris-HCl (pH 6.8) containing 0.1% Triton-X100 and 0.1% 2-mercaptoethanol, freeze thawed twice and homogenised. After centrifugation, the supernatant was assayed for enzyme activity as previously described (chapter 2). The peripheral B cell GTase was assayed twice and the splenic B cell GTase four times in each experiment.

3. Results (Table 8.1, Reference 275).

<table>
<thead>
<tr>
<th>MOUSE STRAIN</th>
<th>PERIPHERAL B CELLS</th>
<th>SPLENIC B CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>lpr/lpr</td>
<td>26±5</td>
<td>73±6</td>
</tr>
<tr>
<td>+/+</td>
<td>15±5</td>
<td>56±5</td>
</tr>
<tr>
<td>CBA/Ca</td>
<td>61±2</td>
<td>63±7</td>
</tr>
</tbody>
</table>

Table 8.1.
Peripheral and splenic (pooled) B cell GTase activity (mean cpm/mg protein.10^5±SEM of 2 experiments) from 37 MRL lpr, 37 MRL +/+ and 60 CBA/Ca mice.

In two experiments using pooled B cells from 37 MRL lpr/lpr, 37 MRL +/+ , and 60 CBA/Ca mice, the mean peripheral B cell GTase activity of both MRL lpr/lpr (26±5 mean cpm/mg protein.10^5 ± SEM) and MRL+/+ (15 mean cpm/mg protein.10^5±SEM) mice was lower than the B cell GTase activity of CBA/Ca mice (61±2 mean cpm/mg protein.10^5±SEM; p<0.005 and p<0.0001 lpr & +/+ respectively) There was no difference between the peripheral B cell GTase activities of the MRL strains.

Also, there was no difference in splenic GTase activity between the MRL mice (lpr: 73±6 & +/+: 56±5 mean cpm/mg protein.10^5±SEM) and CBA/Ca mice.
(63±7 mean cpm/mg protein.10^5±SEM), whereas there was a reduction in the MRL+/+ splenic GTase activity when compared to the MRL lpr strain (p<0.05).

When the peripheral and splenic GTase activities were compared within each mouse strain, the peripheral B cell GTase activity was reduced in both the lpr and +/+ strains when compared to the splenic GTase activity (p<0.001), whereas there was no difference between peripheral and splenic B cell GTase activities in the CBA/Ca strain.


These results indicate that peripheral B cell GTase activity is reduced in MRL mice, with and without the recessive lpr gene, when compared to the non-autoimmune CBA/Ca mouse strain and when compared to their own splenic B cell GTase activities. No such differences between peripheral and splenic B cell GTase activities were apparent in the CBA/Ca mice. Interestingly, both the peripheral and splenic B cell GTase activities were lower in the MRL+/+ strain when compared to the lpr strain.

These results would imply that reduced B cell GTase activity is neither directly responsible nor caused by the autoimmune disease and arthritis associated with the MRL mouse.

That the GTase activity is reduced only in the peripheral and not the splenic B cells of the MRL mice, whereas activities in both compartments are similar in the CBA/Ca mice, may suggest that the B cells sampled in the peripheral blood of the MRL mice are trafficking from areas associated with the development of autoimmune disease, for example gut associated lymph tissue or synovial tissue, rather than the possibility that peripheral B cells are dormant.

The reason that GTase activity is reduced by a larger amount in both compartments in the +/+ strain in comparison to the lpr strain is not readily apparent and may be related to the genetic lineage of the mouse families studied and hence inconsequential in terms of the development of pathology. The development of disease in the lpr mice may, however, result in protective mechanisms being triggered in the immune system with the result that abnormal B cells, with low GTase activity, are targeted and destroyed.

Further investigations are now desirable to determine what role, if any, reduced GTase activity plays in the development of disease in the MRL mouse model of autoimmunity. Such experiments should include a longitudinal study of B cell GTase activity in relation to IgG glycosylation and the development of disease and whether similar defects are apparent in other mouse models of
autoimmunity (ie: NZB/W, BXSB), and the parent strains of the MRL mouse (C57BL/6J, C3H/Di, AKR/J & LG/J).
CHAPTER 9.

DISCUSSION.

The initial experiments set out to determine whether those reagents used by other workers were necessary for a GTase catalysed reaction and whether different acceptors (ovalbumen and N-acetylgalcosamine) could be used to receive galactose in this system. The necessity for manganous ions and cold UDP - galactose was verified, and it was determined that 1μCi of radioisotope per reaction was necessary to ensure that the assay was sufficiently sensitive to cope with variation in amount of enzyme derived from human lymphocytes. The use of a protein carrier (BSA) was observed to be of no value.

Preliminary lymphocytic assays confirmed that the GTase assay was reproducible and reliable. The coefficient of variance was 4.4 %, but increased to between 13.4 % and 15.7% after storage for between three and six months. These latter results indicate that enzymatic degradation was probably occurring. Confidence that the readings of enzyme activity were being obtained from the straight line rather than the plateau part of the GTase activity curve was provided by the observation that addition of supplemental GTase (bovine) increased the counts per minute obtained 10-fold.

GTase activities in serum and plasma were also demonstrated, but no correlation was found between lymphocytic and serum GTase activities, and it was not thought useful to continue further measurements of serum GTase in the rheumatoid arthritis study.

Further confirmation of the assay's specificity was obtained using UDP, an end product of the reaction, as an inhibitor. Linear inhibition of GTase activity was obtained, in both commercial bovine GTase samples and human lymphocytic GTase preparations, to just less than 50% with a 10-fold increase in inhibitor concentration (between zero and 10 mmoles/l). 100 mmoles/l of UDP in later experiments totally inhibited the enzyme reaction. This result indicates that either large fluctuations of UDP are necessary for physiological control of intracellular GTase, or that small changes in GTase activity exert sufficient physiological influence.

These initial experiments therefore confirmed that a reproducible and reliable GTase assay had been devised that could be used for inter-laboratory comparison of activities if the specific activity of the radio-label was kept constant.
The control population used in this study was examined in detail. No sex differences were observed in B and T cell GTase activity and in all but three individuals IgG glycosylation fell within two standard deviations of the mean for a normal population. These three individuals did not have abnormal GTase levels. Simple and polynomial regression analysis between lymphocytic GTase and the individual's age was determined and no association found. This was not entirely unexpected as the normal age-related IgG-Fc glycosylation change (113) is the product of perhaps frequent (minor) fluctuations of lymphocytic GTase activity. Age matching was however carried out in the RA and chronic lymphocytic leukaemia analyses, as patient numbers were sufficient, but other diseases were compared to the total control population.

Interestingly, a positive correlation between GTase and IgG glycosylation was found in the normal population, which would imply that a feedback mechanism may exist causing an increase in GTase activity as IgG glycosylation decreases. Initial GTase activity data, from patients with RA, showed that B cell GTase activity was reduced in RA and also inferred that monocyte GTase was raised in RA. However, total white cell GTase activity decreased from a raised level on removal of monocytes. On studying larger patient numbers, this observation was confirmed and the reduced GTase activity was also found in the T cell enriched preparations. Examination of additional patients with RA revealed a positive correlation between B and T cell GTase. This may be an important finding in the context of this research, as, although the study set out to link GTase changes with IgG-Fc galactose changes, other carbohydrate changes could of course also be occurring within different glycoproteins. For example, HLA DR antigens (247), Thy-1 glycoprotein (248), Von Willebrands factor, factor viii, tissue plasminogen activator (249), human neutrophil Fc receptor (250), the T cell receptor (251), and murine IgE receptor (252) contain abundant amounts of N-linked oligosaccharides and variation in N-glycosylated structures has been observed between these glycoproteins (247,248). RA has also been shown to be associated with elevated levels of fucosylated haptoglobins (253). It remains to be seen whether the reported galactosylation defect in RA intestinal mucin (140a) is associated with a concomitant intestinal GTase defect towards mucin as an acceptor.

The lymphocytic GTase data would suggest that the RA galactosylation defect is synthetic in nature, and this hypothesis is given added weight by the following additional findings. Firstly, cultured RA B cells synthesize agalactosyl-IgG when stimulated by pokeweed mitogen (254), whereas normal B cells do not. Secondly, inflammation in early Lyme disease (eg: carditis, meningitis and
erythema migrans) is not associated with defective glycosylation, whereas the development of arthritis in these patients is (140). Finally, early synovitis is only associated with increased G(0)% in those patients that subsequently go on to develop RA (255).

On splitting the RA population into groups, dependent upon drug treatment, it was noted that those patients taking sulphasalazine (SASP) had higher mean levels of GTase than those on no medication / non steroidal anti-inflammatory drugs, or other second-line agents. This effect was not due to disease activity and was therefore presumed to be related to the actions of SASP itself. SASP has been shown to have potential to cause these effects.

SASP has a suppressive effect on mitogen induced transformation of murine spleen cells (205), guinea pig and human lymphocytes (229,256), and the phytohaemoglutinin stimulated increase in messenger RNA levels have been shown to be similarly affected (257).

B cell function in vivo is also affected by SASP (229,258). However, there is no evidence that it induces a reduction in total B and T cell numbers (259), nor antibody dependent cell mediated cytotoxicity (257), although there appears to be a reduction in circulating activated lymphocytes (229). It has been suggested that these effects might be caused by SASP induced folate depletion (260), which may inhibit DNA synthesis. Furthermore, the random migration of neutrophils is inhibited by SASP (261) and the molecule has been shown to act as an antioxidant (262) and inhibitor of the synthesis of lipoxygenase products (263,264). In this respect, iron is thought to play a role in the generation of free radicals (265), which may be facilitated by the presence of transferrin receptors on the surface of activated B cells (205). SASP may bind free iron and inhibit this chain of events (205).

There is little data on the T cell effects of SASP, but the lack of reduction in suppression of mitogen stimulation by indomethacin in cell culture has led to the suggestion that helper-inducer T cells could be a prime target for SASP suppression (205).

No correlation between disease activity and GTase activity was found. This may be due to subclinical disease activity resulting in continued aberrant cellular dysfunction and the fact that GTase activity is a potentially sensitive measure of disease activity that cannot readily be compared with a crude measure of the clinical end product of disease. It may be that correlation with the development of joint erosions is a more suitable measure of disease activity. Also, in future studies it would be important to differentiate between disease inactivity due to natural disease history and that due to drug therapy. Such studies would require
RA patient populations in disease remission on no medication and some commencing or stopping therapy. This may be especially pertinent in the case of sulphasalazine. In this respect, an initial longitudinal analysis of fluctuations in glycosylation parameters in RA patients was made. Twelve patients were investigated over an 18-month period and a total of 52 observations were obtained for analysis. The 12 patients were divided into three groups on the basis of disease activity and whether or not sulphasalazine was being prescribed. Patient compliance was checked by regular following up, together with detailed questioning to ascertain drug effects, and was thought to be good. Some tentative observations were made associating changes in GTase activity with G(0)% and clinical activity. Firstly, a temporal relationship between glycosylation parameters and clinical activity was found to be present in three patients with active RA who were not taking SASP. Secondly, SASP tended to maintain GTase levels, but this did not always result in changes in disease activity nor G(0)% levels, and may therefore obscure any temporal relationship that may be present. Thirdly, cellular dysfunction may still be present in a clinically inactive patient who is maintained on second-line anti-rheumatic agents, and this was shown by abnormal intracellular GTase activity in some of these patients.

A number of difficulties were noted to be inherent in this study and its interpretation, namely the lack of knowledge of normal fluctuations of GTase and IgG glycosylation and how GTase is itself regulated within the cell. A standardised three week study with all patients being investigated at the same regular interval would help solve, in part, some of these problems. Data from patients with Mycobacterium tuberculosis infection, Crohn’s disease, SLE, and chronic lymphocytic leukaemia (CLL) were obtained. In tuberculosis infection, GTase and IgG glycosylation was always abnormal in patients with active disease (i.e. those on no medication or seen within 8 days of starting treatment). In the small number of patients followed up (3), GTase activity was seen to rapidly return to normal with treatment, whereas IgG glycosylation seemed to lag behind. This would imply that the relationship between lymphocytic GTase and IgG glycosylation is not simple. It may be that fluctuations in different GTase isoenzymes, being involved in the glycosylation not only of IgG but also a variety of other glycoproteins can occur and may explain why the overall cellular level of GTase can be within control limits, when IgG glycosylation is abnormal.

In the study of 12 patients with Crohn’s disease, GTase was found to be low only in active disease. However, GTase activity did not seem to increase with
improvement in clinical state in those patients followed up, and this may be indicative of subclinical disease activity. In this context it is of note that there is reduced IgA and enhanced IgG production by intestinal mononuclear cells isolated from patients with inflammatory bowel disease (266), and it would be of interest to determine whether this alteration in isotype resulted in the production of agalactosyl-IgG. The observation of continued glycosylation abnormality despite treatment, together with the outward signs of clinical quiescence has also been made in the context of the RA studies and it is interesting that in TB, a disease known to cause glycosylation changes, cure results in the normalization of glycosylation parameters.

It is therefore not surprising that when data from the 12 patients studied with SLE were analyzed, a significant decrease in both B and T cell GTase activity was noted in most patients (11/12) irrespective of disease activity, and the medication being taken. In contrast, the IgG glycosylation of these patients concurred in part with that previously observed, in that the presence of agalactosyl IgG (3/12) was not as apparent as that found in a RA population, but agalactosyl IgG was only noted in a minority (1/3) of the lupus patients with Sjögren’s syndrome. This may imply that in SLE glycoprotein changes do occur and GTase activity is affected, but the end result is more frequently manifested by changes in glycoproteins other than IgG, although analysis of total carbohydrate content within the IgG molecule itself in SLE has been reported to have low galactose levels (120,121), which could indicate carbohydrate variation in the Fab region of IgG.

Putting this together in the context of RA, tuberculosis infection, Crohn’s disease and SLE, one may hypothesize that not only can sub-clinical disease cause fluctuations of GTase activity, presumably indicative of continuing cellular dysfunction, but low levels of total GTase activity need not result in abnormally glycosylated IgG. Fluctuations of total GTase may be indicative of changes in glycosylation of other glycoproteins which may appear on the cell surface (e.g. T cell receptor) or be secreted (e.g. cytokines). Hence, the clinical manifestations of the disease may depend upon the glycoprotein(s) involved, which may not be readily apparent by measuring total cellular GTase activity.

These findings become all the more intriguing when B cell GTase activity from patients with CLL is considered. A significant decrease in GTase activity was noted when compared to a control population with the same age range. This may imply that GTase activity can also vary within B cell subsets and it may be pertinent that in this case the B cell subset concerned (CD5+) has been implicated in the generation of autoantibodies (267). This finding should now
be tested using CD5 positive B cells from RA patients, as the presence of cell surface CD5 does not necessarily infer functional similarity between RA and CLL B cells.

Hence, it may be hypothesised that specific isoenzyme fluctuations of GTase may result in variation of galactose content in different glycoproteins, including IgG, culminating in a variety of pathological events. This may be accomplished either by fluctuations in intracellular GTase levels or through the proliferation of B cell subsets that have predefined GTase activity. This would infer a complex cellular communication system, possibly directed by antibodies associated with cell surface determinants, that may include GTase itself. In part, this hypothetical situation has been observed. Galactose α1-3 Galactose (Gal α 1-3 Gal) residues are expressed on the cell surface of human malignant cells, but not normal cells, and anti-Gal α 1-3 Gal antibodies, are capable of inhibiting the cellular attachment of these malignant cells to other cells (268) and hence their ability to metastasize.

Fluctuations in lymphocytic GTase activity in disease have been documented in some detail. In an attempt to investigate how these fluctuations occur two studies were completed. Firstly, the presence of an intracellular inhibitor was looked for by attempting to inhibit commercial bovine and human milk GTase with RA lymphocyte extract. Secondly, specific polymorphisms within the gene encoding the GTA protein were looked for, to determine whether a genetic abnormality existed.

Using normal GTase from human lymphocytes, bovine milk and human milk, no inhibition of activity was detected when mixed with a RA lymphocyte extract. In one experiment synergism was noted when both B and T lymphocytic GTase was mixed with human milk GTase, using N-Acetyl glucosamine as the acceptor. But this was found both with RA and normal preparations. This might indicate that a co-factor may be present in human lymphocytes, not present in the recipe used in the GTase assay, that is capable of boosting the activity of human milk GTase or that galactose is being transferred to endogenous (lymphocytic) acceptors by the purified GTase.

On restriction enzyme digestion of RA and normal lymphocytic DNA, no gross structural changes in the GTA gene were seen and abnormalities of the GTase catalytic component are also unlikely to be responsible for reduced GTase activity as the degree of galactosylation fluctuates with disease activity (114) with normal galactosylation being re-established during remission.

The inflammatory process occurring in RA is known to lead to the production of high levels of cytokines, such as tumour necrosis factor (TNF) and IL-6
Several transcription factors are now known to be cytokine-inducible and in some cases several different transcriptional factors can be induced by a single cytokine. For example, IL-1 is known to induce AP-1, NFkB and NF-IL-6 which bind to cis-acting elements on IL-2, Igk and IL-6 gene loci respectively (271). TNF has also been shown to induce several trans-acting factors (271). IL-1, TNF and hepatocyte stimulating factor are all able to influence the glycosylation of acute phase proteins (272). It will be important to establish whether such cytokines are also capable of inducing alterations in IgG galactosylation via transcriptional regulation of GTase genes.

Using other restriction enzymes, RFLPs were observed in both normal and RA DNA using Bgl II, which may indicate that the GTA gene may exist as different polymorphic alleles, and it may be that polymorphisms of the GTA gene locus may contribute to the RA phenotype. It would now seem appropriate for the level of expression of the GTA gene (ie. amount of messenger RNA) to be determined in a RA population and in normal individuals with low GTase and deglycosylated IgG. Furthermore, now that the gene for the GTase catalytic component itself has been cloned and genomic clones produced (Dr L Mengel-Gaw, personal communication), re-probing the digested DNA blots can be achieved with, perhaps, a more relevant gene probe.

It has previously been suggested that isoenzymes of GTase are necessary to transfer galactose to different acceptors (57) and in this discussion it has been suggested that isoenzyme fluctuations may be important in determining where glycoprotein changes occur and what disease results. To test this hypothesis, in part, GTase from normal individuals and RA patients was taken and the ability to transfer galactose to a panel of five different acceptors determined. A decrease in enzyme activity was noted using each acceptor, but a significant decrease comparable to that found with ovalbumin was only noted in asialo-mucin. Although it is appreciated that both these acceptors are O-linked (IgG is N-linked at Fc), a difference has been shown to exist indicating that individual GTase isoenzyme fluctuations may occur. GTase activity changes have been detected in bladder transitional cell carcinoma, where a difference in activity towards fetuin was observed (273), but other workers (274) have found that no difference exists in GTase activity towards GlcNAc and asialo-orsomucin, but a 50% reduction in activity was detected in B cell GTase when deglycosylated human IgG was used as an acceptor in RA patients. Furthermore, liver cell homogenates from the same patients could glycosylate agalactosyl-IgG, inferring that it is a specific GTase in RA B cells that is defective or the levels reduced.
The future use of IgG-Fc, in varying degrees of glycosylation, as an acceptor may yield some interesting data, and isoelectric focusing of GTase, from various sources, may provide some quantitative evidence that isoenzyme variation occurs. So far only peripheral human lymphocytes have been studied and it could be that peripheral B cells are dormant and unimportant in the context of disease. To help answer this question, the MRL-lpr/lpr mouse, a model for RA (275), was used. As determined in the initial experiment, the GTase assay is insensitive when less than half a million cells are used. GTase from pooled peripheral or splenic B cells was, therefore, taken and assayed, in two identical experiments, from MRL-lpr/lpr (arthritic), MRL+/+ (non-arthritic) and CBA/Ca (non-autoimmune) strains of mice all of ages when arthritis was present in the lpr/lpr strain. Splenic B cell GTase activity was found to be similar in both the MRL strains and the CBA/Ca control strain, whereas enzyme activity was reduced in the peripheral B cells only in the MRL strains, indicating that this reduction is due to the disease rather that dormancy. This may imply that firstly, reduced GTase activity does not cause arthritis per se, as seen also in the human experiments, and secondly, there may be a specific abnormality within the peripheral B cells in RA and what is happening is that an increased number of cells, perhaps from a specific subset, are being picked up in the periphery that are trafficking from inflamed joints, for example. In experiments with adjuvant arthritis in Lewis rats (276) reduced IgG-Fc galactose has also been reported together with a concomitant decrease in GTase activity.

Our knowledge of oligosaccharide structure and function has increased tremendously over the past two decades (see introduction) and there is ample evidence that glycoproteins have the potential to play an important part in the normal cellular communication network that may be involved in the function of the immune system. Changes in this communications system may be pivotal to disease pathogenesis.

The purpose of this study was to determine whether changes were present in the activity of GTase found in lymphocytes in RA and other diseases. This was achieved, and a number of hypotheses have been generated within this discussion to account for the data, which can be put together as follows:

1. A variety of oligosaccharide changes can occur in pertinent glycoproteins and may be related to the development of specific diseases.

2. Changes in these oligosaccharides may be mediated by variation in different carbohydrate transferases, with more subtle variation occurring in isoenzymes within these families. The situation described in the lupus patients
studied may be an example of this, where there is a profound reduction in GTase activity which is generally not associated with an increased G(0)%. It may be that the reduced GTase activity in these patients results in abnormal glycosylation of another glycoprotein, complement for example, associated with the pathogenesis of SLE.

3. Regulation of carbohydrate transferases may be achieved by antibodies, such as anti-GTase, which may change intracellular GTase activity via cell surface receptors or cause dominance of GTase negative B cells as a result of cytotoxicity towards GTase positive B cells. Proliferation of GTase negative cells may also occur by other mechanisms, such as cytokine inhibition of transcription of the GTase catalytic gene.

A hypothetical scheme of these events is depicted in figures 9a & b, where the heterogenous nature of rheumatic autoimmune diseases is highlighted by the large number of predisposing factors and disease 'triggers' that are associated with these diseases (see also Introduction). Following an initial immunological insult, the normal regulatory homestatic immune mechanisms breakdown, resulting in, amongst other things, a reduction in carbohydrate transferase activity. Any number of transferases may be affected, and subtle variation is achieved by isoenzyme changes. A change in glycosylation pattern of pertinent glycoproteins will result, which sets off a chain of immunological events in relation to the abnormal glycoprotein. For example, immune complexes may form due to abnormal IgG-Fc glycosylation, autoreactive T cells may be generated due to TCR abnormalities and antibodies against glycoprotein may be formed that cross react with other cell surface determinants, HLA class II molecules for example. The end result is a spectrum of clinical disease, which may perpetuate the cycle of events by providing further immunological insults to the system.

The two important observations that, however, are necessary to help complete this mosaic (267) are firstly, proof that glycosylation changes are not merely secondary inflammatory phenomena, and secondly, to determine the physiological role of glycoproteins in normal cellular function.
Figure 9a.
Part 1. Glycoprotein involvement in rheumatic autoimmune disease. A hypothetical chain of events leading from a disease predisposition to an event triggering the disease and leading to the generation of carbohydrate transferase abnormalities.
Part 2. Glycoprotein involvement in rheumatic autoimmune disease. Carbohydrate transferase abnormalities may lead to specific glycoprotein changes, which may be associated with distinct diseases (i.e., SLE and RA). Subtle variation within each glycoprotein family may result in disease variation (i.e., Felty's syndrome, Sjögren's syndrome & mixed connective tissue disease). This chain of events may be self perpetuating or further triggered by an otherwise inconsequential immune interaction.
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REduced b-cell galactosyltransferase activity in rheumatoid arthritis

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Summary

Autosensitisation to IgG may be important in the pathogenesis of rheumatoid arthritis and could be related to reduced glycosylation of the oligosaccharides in the C2 region of serum IgG. The activity of galactosyltransferase, the enzyme that catalyses the addition of galactose to the oligosaccharide chains, was measured in the circulating B cells of seventeen patients with classic rheumatoid arthritis. It was significantly lower than that of a group of eleven controls (p < 0.001) or of nine age-matched controls (p < 0.01). In contrast, the enzyme activity of the T cells was within the range of that in nine age-matched controls, and enzyme activity in monocyte-rich mononuclear-cell populations was higher than in controls, possibly reflecting stimulation of the monocytes in rheumatoid arthritis. These findings suggest that galactosyltransferase may regulate the degree of glycosylation during IgG synthesis and could therefore be implicated in the rheumatoid inflammatory process.

Introduction

It has been argued that IgG autosensitisation may have a central role in the pathogenesis of rheumatoid arthritis, and it is of particular importance that galactosylation abnormalities in the serum IgG of patients with this disorder have been reported.

The Fc portion of IgG contains the second and third globular domains of the constant regions of the two IgG heavy chains (C2 and C3 domains). The pair of C3 domains are intimately associated with each other by non-covalent interactions, but the two C2 domains are spatially separated by a bridge formed by the two oligosaccharides (Figs 1 and 2), each attached to an asparagine residue at position 297 of the heavy chain. The two side chains in each oligosaccharide unit are linked to the mannose in the main core by \( \alpha(1-3) \) and \( \alpha(1-6) \) glycosidic bonds, respectively. The galactose residue on the \( \alpha(1-6) \) arm appears to fit into a lectin-like pocket (P) on the surface of the C2 domain. One of the \( \alpha(1-3) \) arms always lacks galactose.

\[ \text{Gal} \quad \text{GlcNAc} \quad \text{Man} \quad \text{Neu5Ac} \]

**Fig. 1—Role of two Fc oligosaccharides (\( \bullet \)) in holding apart two C2 domains.**

which means it can interact with a sugar in the opposing oligosaccharide (arrowed on Fig 2), so keeping the two C2 domains apart. The extent of galactosylation of the three remaining chains varies. We have found (Parekh RB et al, unpublished) that the percentage of oligosaccharide chains completely lacking galactose falls with age from 1 year to 25 years then increases.

The finding of reduced galactosylation of IgG in patients with rheumatoid arthritis compared with normal individuals and the lack of evidence of a postsynthetic degradative process stimulated our study of the galactosyltransferases. These enzymes are normally golgi-membrane-bound and in the lymphocyte catalyse the addition of galactose to N-acetylglucosamine during the biosynthesis of the carbohydrate moieties of glycoproteins and glycolipids.

We have measured the enzyme activity in peripheral-blood lymphocytes by following its catalysis of the reaction between UDP-galactose and the acceptor sugar N-acetylglucosamine, present as a non-reducing terminal monosaccharide of a carbohydrate side chain in the glycoprotein ovalbumin, in the belief that a reduction in enzyme activity might cause the reduced IgG galactosylation characteristic of patients with active rheumatoid arthritis.

\[ \text{Gal} \quad \text{GlcNAc} \quad \text{Man} \quad \text{Neu5Ac} \]

**Fig. 2—General structure of one of IgG oligosaccharides located at C2 domain.**

GlcNAc = N-acetylglucosamine; Man = mannose; Gal = galactose; Neu 5Ac = sialic acid (presence on Fc chains is uncertain); ASN = asparagine.

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**Patients and Methods**

The study population consisted of patients with classic rheumatoid arthritis and patients with no clinical or serological evidence of autoimmune disease. The whole mononuclear-cell populations analysed were obtained from nineteen female patients with rheumatoid arthritis (mean age 59.4 years, range 39–82 years) and nineteen age-matched control patients (mean age 58.2 years, range 25–77 years; sixteen female, three male). The monocyte-depleted mononuclear-cell populations came from ten rheumatoid arthritis patients (mean age 53.5 years, range 23–72 years; eight female, two male), and ten age-matched control patients (mean age 50.9 years, range 20–72 years; eight female, two male). Isolated B-cell and T-cell populations were obtained from seventeen rheumatoid arthritis patients (mean age 62.6 years, range 41–80 years; all female), and eleven control patients (mean age 53.4 years, range 24–75 years; eight female, three male). Nine rheumatoid arthritis patients (mean age 60.3 years, range 41–75 years) were age-matched with controls (mean age 59.5 years, range 33–75 years; seven female, two male).

Peripheral blood was obtained by venesection and, when required, depleted of monocytes by incubation at 37°C with carbonyl iron, followed by separation with a magnet. Peripheral-blood lymphocytes were then separated by means of ‘Ficoll-Hypaque’. Cell purity was assessed by May-Grunwald-Giemsa staining, the lymphocytes accounting for at least 95% of the total population. The B cells and thymus-derived T cells were further separated by rosetting with neuraminidase-treated sheep red blood cells. Assessment of cell purity by rosetting showed at least 95% rosettes in the T-cell population and no more than 10% rosettes in the B-cell population.

The lymphocytes were incubated at 37°C for 1 h in balanced salt solution containing 10% fetal calf serum, then resuspended in 100 mmol/l “tris”-HCl (pH 6.8) containing 0.1% ‘Triton-X100’ (Sigma) and 0.1% 2-mercaptoethanol (BDH), frozen and thawed twice, and homogenised in a glass homogeniser. The homogenate was centrifuged at 10 000 g for 10 min, was assayed for enzyme activity. The standard incubation medium for the assay contained 0.5 mmol/l ATP (Sigma), 20 mmol/l manganous chloride (BDH), 100 μmol/l UDP-galactose (Sigma), 1 μCi UDP-[3H]galactose (Amersham International), and 1 mg ovalbumin in 100 mmol/l tris-HCl to a total volume of 100 μl, after addition of 20 μl enzyme extract. The mixture was incubated for 2 h in a shaking water bath at 37°C. The reaction was stopped by the addition of 2 ml 1% phosphotungstic acid in 0.5 mol/l hydrochloric acid. The precipitate was filtered onto Whatman GF/A glass microfibre filters, washed, dried, and counted. Protein concentration was estimated (Bio-Rad Laboratories), and enzyme activity calculated from a standard curve with bovine galactosyltransferase (Sigma).

**Results**

The galactosyltransferase activity of whole mononuclear-cell preparations was significantly higher in rheumatoid arthritis patients than in age-matched controls (mean [SEM] 100.4 ± 18.0 vs 61.6 ± 5.0 nmol/h/mg protein; p < 0.001, Mann-Whitney U-test, two-tailed; fig 3), whereas in monocyte-depleted mononuclear-cell preparations there was no significant difference between patients and controls (57.6 ± 6.4 vs 52.3 ± 6.0 nmol/h/mg protein; fig 3). In isolated B-cell preparations galactosyltransferase activity was significantly lower in rheumatoid arthritis patients than in the controls for the whole group (25.4 ± 7.4 vs 81.1 ± 10.6 nmol/h/mg protein; p < 0.001) and in the age-matched group (26.2 ± 10.6 vs 83.6 ± 12.5 nmol/h/mg protein; p < 0.001; fig 4). In isolated T-cell preparations, though the galactosyltransferase activity was significantly lower in rheumatoid arthritis patients than in the controls for the whole group (25.4 ± 7.4 vs 81.1 ± 10.6 nmol/h/mg protein; p < 0.001), the difference for the age-matched group was not significant (57.9 ± 24.2 vs 83.5 ± 17.8 nmol/h/mg protein; fig 4).

**Discussion**

Our finding that galactosylation tends to vary with age (unpublished) suggests that some of the processes involved in rheumatoid arthritis are age related. The frequency of rheumatoid factor increases with age. However, by careful age-matching in this study, we have shown that...
THE HUMAN THYMUS CONTAINS A NOVEL POPULATION OF B LYMPHOCYTES

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Summary A study of thymus glands from fetuses, newborn babies, children, and adults has shown a prominent intramedullary B-cell population. These cells have a distinctive phenotype, show evidence of activation, and specifically cluster around Hassall’s corpuscles. The thymus can no longer be regarded as an organ exclusively concerned with T-cell function.

Introduction

The structure and function of the thymus have been intensively investigated since Miller demonstrated the central role of this organ in the generation of the immune response some 25 years ago. Investigations of the thymic microenvironment have concentrated largely on T-cell ontogeny. Plasmablasts have been described in thymic perivascular sheaths, where B-cell follicles may also be found, especially in patients with myasthenia gravis. The basement membrane of the perivascular sheath separates these follicles from thymic parenchyma, and B cells are not a recognized component of the thymic microenvironment.

Lymphoproliferative disorders that arise in the thymus include T-cell lymphoblastic lymphoma and Hodgkin’s disease of the nodular sclerosing subtype. A distinctive type of primary mediastinal non-Hodgkin’s lymphoma has also been described. This tumour, which appears to arise in the thymus and is commonest in young women, was first thought to be derived from T cells. However, immunohistochemical studies have shown unequivocally that it is a B-cell tumour. It was this finding that raised the possibility that the normal thymus contains a B-cell population and prompted the study described here.

Methods

Thymic tissue was obtained from 16 patients (aged 9 months to 66 years) during open heart surgery. In 12 cases only formalin-fixed paraffin-embedded tissue was available, but fresh tissue was obtained from 4 patients, and this was snap-frozen in liquid nitrogen and preserved at -70°C. In addition, paraffin-embedded thymic tissue removed at necropsy from 15 fetuses (at 15-40 weeks’ gestation) and 3 infants aged 2, 3, and 4 months, respectively, was retrieved from the files of the Department of Histopathology, University College and Middlesex School of Medicine.

Both paraffin-embedded and frozen tissue were stained haematoxylin and eosin. Sections from paraffin-embedded material were also stained for reticulin (Gordon and Sweet) to demonstrate perivascular sheaths. Paraffin sections were immunostained with
The Role of IgG Glycoforms in the Pathogenesis of Rheumatoid Arthritis

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Introduction

Most serum-derived and cell-surface proteins are N-glycosylated, i.e., have oligosaccharides covalently attached to Asn through an N-glycosidic linkage. The structure of these highly branched oligosaccharides is often very complex, since their constituent monosaccharides can be linked in many different ways [28]. Consequently, the potential information encoded into an oligosaccharide via its monosaccharide sequence and three-dimensional structure is considerable. Further, an individual N-glycosylation sequon usually does not contain a single oligosaccharide, but rather a qualitatively and quantitatively conserved population of structures, and this is usually referred to as microheterogeneity. This implies that polypeptides are diversified by glycosylation into populations of glycoproteins differing with respect to the structure and disposition of oligosaccharides on a common polypeptide (i.e., glycoforms). Each glycoform may be involved in different and unique cellular functions (e.g., 'homing', clearance, etc.). It follows that disease-associated changes in the incidence of individual naturally occurring glycoforms, or the generation of new glycoforms, will affect the carbohydrate-dependent functions of a polypeptide. Changes in oligosaccharides may in some cases, therefore, contribute directly to disease pathogenesis. Moreover, the final population of N-linked oligosaccharides carried by a polypeptide is the result of a large number of intra-cellular events, some of which are tissue specific [42]. Therefore, irrespective of the normal functions of the oligosaccharide, a comparative analysis of the N-glycosylation of particular glycoproteins provides an excellent probe for acquired or inherited cell type-specific or tissue-specific dysfunction.

All normal IgG isotype antibodies are glycoproteins carrying predominantly N-linked oligosaccharides [41, 49]. Briefly, human serum IgG carries, on average, 2.8 N-linked oligosaccharides, of which 2.0 are invariably located in the Fc at the conserved N-glycosylation site of Asn 297 (Fig. 1). The additional oligosacchara-
Fig. 1. a A schematic representation of an IgG molecule indicating the positions of conserved N-glycosylation sites (at Asn-297 in the C\textsubscript{2} domains) and non-conserved N-glycosylation sites (in the hypervariable regions (dotted) of the Fab region). The relative size of an immunoglobulin domain and a fully extended N-linked biantennary complex oligosaccharide are similar [4]. Complex-type oligosaccharides present on IgG can be subdivided into an outer arm region (a, a', b, b', c, c'), and the core, which is composed of a trimannosyl unit (d, d', e) and a N,N'-diacyctylchitobiosyl unit (f, g). The 'bisect' GlcNAc (residue i) is linked \( \alpha(1-4) \) and the fucose (residue h) is linked \( \alpha(1-6) \). The arrow between residues e and f indicates the site of interaction between the two oligosaccharides in b and c. b Refined structure at 2.8\( \AA \) of rabbit Fc fragment [54]. The two carbohydrate chains, each attached at Asn 297, differ in conformation and may also differ in sequence and bridge the two C\textsubscript{2} domains. The \( \alpha(1-3) \) arm of the chain (left side) is always devoid of galactose and interacts through its \( \beta(1-2) \)-linked GlcNAc residue (c) with the Man\( \beta(1-4) \) GlcNAc segment of the opposing (right side) oligosaccharide chain (see a). The \( \alpha(1-3) \) arm of the right chain extends outwards between the domains with no apparent steric constraints on its length. A Neu5Ac unit (a') is shown on one \( \alpha(1-6) \) arm only (left). The extent of oligosaccharide heterogeneity in a single crystal is identical to that found in pooled Fc fragments [49], consequently the X-ray data represent the composite structure. c Fc fragment containing oligosaccharides devoid of galactose and sialic acid on each of the \( \alpha(1-6) \) arms. Since these residues in normal IgG are in contact with the surface of the protein (see b), their absence vacates oligosaccharide-binding sites in IgG from arthritic patients and could make the IgG 'sticky' by creating a lectin-like activity. It is not known to what extent the remaining sugar residues remain in contact with the peptide.
IgG Glycoforms in Rheumatoid Arthritis

IgG glycoforms are located in the variable region of the light and heavy chains, with a frequency and position dependent on the occurrence of the N-glycosylation sequon [Asn/Xaa/Ser(Thr)]. Approximately 30 different biantennary oligosaccharides are found to be associated with total human serum IgG (Fig. 2). These are distributed non-randomly between the Fab and Fc. Characteristics of Fc N-glycosylation include the absence of disialylated structures, a low incidence of monosialylated ones (~10%), a low incidence of cores carrying a 'bisecting' GlcNAc, and the absence of galactose on the α1-3 arm of at least one oligosaccharide chain in each Fc. Fab N-glycosylation is characterised by a high incidence of di- and monosialylated structures, and of cores with the 'bisecting' GlcNAc residue. The large number of different structures associated with IgG is not the result of studying a polyclonal population, since a similar heterogeneity is found upon analysis of myeloma and hybridoma IgG [49]. This heterogeneity, therefore, creates a very large number of variants (glycoforms) of each unique IgG polypeptide causing further structural, and perhaps functional, diversification.

Altered N-glycosylation of IgG in Rheumatoid Arthritis

Studies of the N-glycosylation pattern of serum IgG (Fig. 3) have shown that a major difference exists between normal individuals and those with rheumatoid arthritis [36, 41, 43]. Structural analysis indicates that serum IgG in patients with rheumatoid arthritis is not associated with any novel oligosaccharide structures, but rather with an increased number of oligosaccharide moieties whose outer arms lack galactose and terminate in N-acetylgalactosamine [41]. There appears, therefore, to be a shift in the population of IgG glycoforms towards those with a higher content of agalactosyl oligosaccharides. This change in N-glycosylation of IgG is generally measured using one of the following three methods: (1) the use of hydrazine to release IgG-associated oligosaccharides, followed by digestion of these oligosaccharides with exoglycosidases of defined specificities [41, 45]. This method yields important structural information, and also allows the degree of outer arm galactosylation to be probed exactly. That is, the relative incidence of digalactosyl, G(2), mono-galactosyl, G(1), and agalactosyl, G(0) oligosaccharides associated with an IgG sample can be accurately determined. (2) The use of anti-GlcNAc monoclonal antibodies. This provides information only on the relative incidence of agalactosyl oligosaccharides. (3) The use of chemical methods to determine the relative monosaccharide composition of a given IgG sample. This method provides no structural information. Since the relative content of di-, mono-, and agalactosyl structures fluctuates with age and disease activity (see later), this latter method may be occasionally misleading. Of the various methods, therefore, exoglycosidase analysis of hydrazine-released oligosaccharides is the most informative, and remains the method of choice for probing IgG N-glycosylation.

The relative incidence of agalactosyl oligosaccharides, G(0), is an age-related parameter [45], as shown in Fig. 4. Interestingly, G(1) does not vary with age, and G(2) varies inversely to G(0) (data not shown). The relation between G(0) and age determined for unaffected individuals establishes the abnormality of this parameter in patients with adult rheumatoid arthritis (Fig. 5a), and allows an
Fig. 2. Primary monosaccharide sequences of the N-linked oligosaccharides associated with human IgG. The hydrodynamic volume (as measured in glucose units) of each structure (or of its neutral derivative in the case of those sialylated) is indicated, and was determined by comparison with α(1→6) linked glucose oligomer standards [3].

Assessment of the normality or otherwise of this parameter in other diseases. From a study of other autoimmune rheumatological conditions, chronic inflammatory disorders, and diseases with known infectious etiology, it emerges that juvenile rheumatoid arthritis, tuberculosis, Crohn's disease, and systemic lupus erythematosus (SLE) with Sjogren's syndrome (Figs. 5, 6) are also associated with an elevated G(0), whereas the other diseases listed in Table 2 are not [44, 46].
Further, G(0) correlates well with clinical score in patients with adult rheumatoid arthritis (Fig. 7), and also with disease activity (Fig. 6). It is also interesting that during pregnancy, a state commonly associated with remission of rheumatoid arthritis, a significant increase in the galactose content of IgG is observed [48].

A comparative analysis of IgG from serum and synovial fluid (Table 1) showed that in four of five patients with rheumatoid arthritis, the level of G(0) in the synovial fluid was higher than in serum. These data are consistent with an intra-articular synthesis of agalactosyl IgG. A comparison of the N-glycosylation of Fab and Fc fragments derived from total serum IgG of patients with rheumatoid arthritis or from a control group shows that the decreased galactosylation found upon analysis of total serum IgG is largely due to changes in the N-linked oligosaccharides of the Fc [43]. There are also quantitatively minor, but potentially significant differences in Fab glycosylation, and these may be restricted to heavy chain N-glycosylation [43, 49]. At present, the relation between the percentage incidence of G(0) and the percentage incidence of agalactosyl IgG [i.e., IgG(0), an IgG molecule containing two paired Fc-associated oligosaccharides both completely lacking galactose] can only be estimated by assuming random pairing of the two heavy chains [41].

It seems likely that these changes in IgG N-glycosylation can be attributed to a reduction in the β-galactosyltransferase activity in the B lymphocytes of patients with rheumatoid arthritis [2]. A specific β-galactosyltransferase has been reported to be present in B lymphocytes, which transfers UDP-Gal to an asialo-agalactosyl
Fig. 4a, b. The variation in the relative incidence of agalactosyl (a) and mongalactosyl (b) N-linked oligosaccharides on total human serum IgG with age. Dots (a) represent the value of G(0) for each individual. The solid curve (a) is a quadratic regression line of the data (n=2, no other transformations improved the fit), and the solid curve (b) is a linear regression line of the data. Dashed curves (a, b) represent calculated regression lines with 95% confidence intervals.
IgG Glycoforms in Rheumatoid Arthritis

Fig. 5. a Relation of the percentage incidence of agalactosyl monosaccharide sequence to age of IgG from patients with adult onset rheumatoid arthritis. All patients studied had active disease at the time of sampling. The closed circles, •, depict values of G(0) from patients reported previously [44] and the open circles, ○, from [41]. The regression line (dashed) for the patients from both studies is given by the equation \( y = 31.9 + 0.264x \), SD of slope 0.079, \( n = 56 \) and is significantly different \( (P < 0.01) \) from a linear approximation (dots) \( [y = 10.2 + 0.343x, \text{SD of slope 0.041, } n = 111] \) of the function for the normal subjects (Fig. 4) between ages 20 and 70, as tested by the analysis of covariance. The 95\% confidence limits for the fit of the data from normal subjects are indicated by solid lines. b Relation of the percentage incidence of agalactosyl monosaccharide sequence G(0) to age of IgG from patients with tuberculosis, •; Crohn's disease, X; and SLE with Sjogren's, □. All patients were active at the time of sampling. The regression lines for the three diseases were calculated from the following equations; tuberculosis, \( y = 50 + 0.007x \), SD of slope 0.132, \( n = 21 \); Crohn's, \( y = 16.6 + 0.88x \), SD of slope 0.42, \( n = 9 \) and SLE+Sjogren's, \( y = 26.8 + 0.32x \), SD of slope 0.17, \( n = 10 \). All three lines were significantly different \( (P < 0.01) \) from a linear approximation of the G(0) data from normal subjects (Fig. 4). The solid line depicts the regression function for normal subjects generated by the least squares method for the means of ages with \( n > 3 \). The outer bounds of the hatched lines depict the regression functions for ±2 SD of the mean for ages with \( n > 3 \).

IgG [16]. The affinity of this enzyme for UDP-Gal in the B lymphocytes of patients with rheumatoid arthritis is lower than in B lymphocytes from a control group. Further, the specific activity of the galactosyltransferase from the B lymphocytes of such patients towards asialo-agalactosyl IgG is found to be reduced to 50\% - 60\% of controls [16]. The galactosyltransferase deficiency also appears to be greater in B lymphocytes than in T lymphocytes or monocytes of patients with rheumatoid arthritis [2]. However, a decrease in the level of \( \beta \)-galactosyltransferase activity of both B and T lymphocyte has recently been found in patients with tuberculosis (J. Axford, personal communication).

Carbohydrate-dependent Functions of IgG

The pathological consequences of the shift in population of IgG glycoforms towards IgG molecules whose oligosaccharides terminate in N-acetylgalactosamine in rheumatoid arthritis, tuberculosis and Crohn's disease can be understood by
Fig. 6. a Variation in the relative incidence of agalactosyl N-linked oligosaccharides on total serum IgG with age in individuals with systemic onset (x), polyarticular onset (Δ), and pauciarticular onset (•) juvenile rheumatoid arthritis. The solid line depicts the regression function for normal subjects (Fig. 4). The values of G(0) for patients were significantly different from age-matched controls \((P < 0.01)\) as determined using an analysis of covariance between the regression lines for the diseased group (dashed) and corresponding age-matched controls (dotted) [45]. b Retrospective analysis of G(0) in patients a separate set from those reported in a with juvenile onset arthritis. Seven of the patients were in remission at the time of the second serum sample. Included are patients with systemic (x), \(n = 6\); poly (Δ) \(n = 3\); and pauciarticular (•), \(n = 1\); presentation. Most patients progressed to symmetrical polyarthritis, \(n = 8\). Disease progression in two patients was limited to the pauciarticular type of arthritis, one symmetric, the other asymmetric. The solid curve represents the age-dependent function for the normal subjects between ages 1 year and 50. c Retrospective analysis of G(0) in a patient presenting with systemic onset juvenile arthritis with progression to symmetric polyarthritis before inactive disease (□) and eventual remission (○).

considering the carbohydrate-dependent effector functions of the Fc and Fab regions of IgG. Carbohydrate-dependent functions of the Fc moiety of IgG all involve interactions with cellular-bound receptors, whereas fluid-phase reactions are independent. For example, aglycosyl and deglycosyl IgG molecules retain the properties of the normal glycosylated molecules in the binding of antigen, protein A, and C1q, and also with respect to C1 activation [30]. However, in such molecules there is complete loss of binding to monocyte and macrophage Fc receptors [30, 39], the ability to induce cellular cytotoxicity is reduced [39],
Table 1. Relative incidence of agalactosyl oligosaccharides in IgG isolated from serum and synovial fluid of patients with rheumatoid arthritis

<table>
<thead>
<tr>
<th>Disease activitya</th>
<th>Age</th>
<th>G(0) percentb</th>
<th>Serum</th>
<th>Synovial fluid</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>38</td>
<td>33</td>
<td>39</td>
<td>25 ±4</td>
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<td>4c</td>
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<td>I</td>
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<td>26 ±8</td>
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<td>A</td>
<td>53</td>
<td>48</td>
<td>48</td>
<td>26 ±8</td>
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<tr>
<td>A</td>
<td>63</td>
<td>43</td>
<td>50</td>
<td>32 ±7</td>
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<tr>
<td>A</td>
<td>80</td>
<td>49</td>
<td>51</td>
<td>n.d.</td>
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a A: Active; I: inactive  
b Values of G(0) are accurate to < 1%  
c Normal values are determined for 5-year intervals from the G(0) vs age data (Fig. 4a)  
n.d. = Not determined

complexes with antigen fail to be eliminated rapidly from circulation [39], and feedback immunosuppression is lost [20]. Serial lectin chromatography of native, normally glycosylated IgG has confirmed that there exists within the total IgG population a range of affinities for monocyte Fc receptors which is independent of subclass, but correlates with Fc N-glycosylation (i.e., different glycoforms) [32]. This indicates clearly that not only the degree but also the nature of Fc N-glycosylation is of biological relevance.

The N-linked oligosaccharides of the Fab moiety of IgG tend to influence solubility/aggregation phenomena. Naturally occurring monovalent antibodies (i.e., non-precipitating) are formed by N-glycosylation of only one of the Fab arms (asymmetric glycosylation). These endo-β-N-acetylglucosaminidase-H sensitive oligosaccharides (and, therefore, putatively of the oligomannose or hybrid classes) act as combining site 'blockers' to render the molecule functionally univalent [12, 29, 31]. Non-precipitating univalent antibodies are known to have enhanced cytotoxic properties as compared to normal bivalent precipitating antibodies [10]. The presence of an oligosaccharide with non-reducing terminal mannose on the non-antigen binding Fab arm may have an additional role in acting as a ligand for

Fig. 7. Relation of the percentage incidence of agalactosyl monosaccharide sequences from IgG of patients with adult onset rheumatoid arthritis to clinical score. The clinical score (mean ± SD), 1 = inactive/mild, n=3; 2 = moderately active, n=7; and 3, severely active, n=4, was determined prospectively for the patients as reported elsewhere [44].
Table 2. Diseases in which the relative content of agalactosyl oligosaccharides in total serum IgG has been determined

<table>
<thead>
<tr>
<th>Diseases associated with normal IgG (0)</th>
<th>Diseases associated with increased IgG (0)</th>
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<tbody>
<tr>
<td>Primary SLE</td>
<td>Rheumatoid arthritis</td>
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<tr>
<td>Primary Sjogren’s</td>
<td>Juvenile-onset RA</td>
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<td>Myositis</td>
<td>Tuberculosis</td>
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<td>Scleroderma</td>
<td>Crohn’s disease</td>
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<td>Psoriatic arthropathy</td>
<td>SLE plus Sjogren’s</td>
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<td>Gouty Arthritis</td>
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<td>Parvovirus infection</td>
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<td>Glandular fever</td>
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the serum mannose-binding protein which activates complement through the classical pathway [24].

The aggregation of human IgG into complexes of various sizes is also critically influenced by the $N$-glycosylation of the Fab moiety of IgG. For example, the cryoglobulin and cold agglutinin properties of certain monoclonal IgM and IgG molecules have been shown to arise from sialylated $N$-linked oligosaccharides located on the Fab [34, 61]. A quantitative analysis of naturally occurring aggregated or self-associated IgG and monomeric IgG showed that aggregated IgG carries an average of 3.5 $N$-linked oligosaccharides per molecule (2 chains on the Fc and 1–2 chains on the Fab), but the monomeric IgG carries an average of 2.2 chains. Comparative analysis of the Fab associated $N$-linked oligosaccharides of aggregated and monomeric IgG showed a ‘selection’ for particular disialylated oligosaccharides in polymeric IgG [47].

In principle, therefore, one would expect the observed changes in the degree of outer arm galactosylation of IgG oligosaccharides characteristic of rheumatoid arthritis, tuberculosis, Crohn’s disease, and SLE with Sjogren’s syndrome to cause pathological effects through any one or more of the following mechanisms: first, through altered interactions with monocyte Fc receptors. Interaction with any such receptor that requires Fc oligosaccharides to retain galactose would be diminished, while any interaction requiring exposed non-reducing terminal GlcNAc would be dramatically increased, particularly as a consequence of the pairing of two agalactosyl oligosaccharides in the Fc. Such pairing would create an Fc moiety possessing four, possibly unrestrained, non-reducing terminal $N$-acetylglucosamine residues (see later), which could interact with a variety of cellular receptors against
GlcNAc. Second, a knowledge of the Fc crystal structure [54] suggests that the loss of outer arm galactose would have significant structural consequences, which may lead to immune-complex formation through one of several mechanisms. These mechanisms and the possible consequences of the increased exposure of non-reducing terminal N-acetylglucosamine residues are discussed in detail below.

Immune-complexes and Glycosylation

In rheumatoid arthritis it has long been postulated that auto-sensitisation to IgG may play a pivotal role in the pathogenesis of the disease, and immune complexes are formed through the binding of the IgM, IgG, or IgA rheumatoid factors to the constant region domains of IgG molecules. The immunogenic site on IgG has been localised to the Fc moiety [38], but there is no evidence for amino acid changes in the Fc of this IgG. Data on the carbohydrate composition of IgG present in the intermediate complexes isolated from the serum of patients with rheumatoid arthritis invariably show an increased level of sialic acid (usually Fab-associated) as compared to normal serum IgG [19, 23], and decreased content of Fc-associated galactose [23]. In one case, this sialic acid was shown to occur on light chains, and was crucial for IgG self-association, since its removal by neuraminidase treatment abolished complex formation [23]. In a recent study [48] it was found that serum IgG from patients with rheumatoid arthritis had a lower carbohydrate content and therefore presumably lower Fab N-glycosylation, consistent with the sequestration into complexes of IgG enriched in Fab N-glycosylation. Together, these observations suggest that immune-complex formation in rheumatoid arthritis could involve both Fab N-glycosylation and agalactosyl structures in the Fc [43]. The molecular mechanism whereby these two factors might contribute to IgG auto-antigencity or self-association are not known, but a study of the crystal structure of Fc provides some insight into this. The crystal structure [54] clearly indicates that each N-linked oligosaccharide in the Fc can interact with the protein surface of the Cα2 domain, principally via the NeuNα2→6 Galβ1→4 segment of the Man α1→6 arm (Fig. 1). This is the principle non-covalent protein-oligosaccharide interaction in Fc, and serves to restrain the Fc oligosaccharides, and also to mask certain underlying Fc polypeptide determinants (Fig. 1). It is, therefore, suggested that the change in the degree of occupancy of the Fc carbohydrate-binding site, secondary to decreased outer arm galactosylation of Fc oligosaccharides, could lead to IgG self-association through one of the following mechanisms: first, through the insertion into this vacant site of an appropriate Fab-linked oligosaccharide from another IgG molecule; second, through the interaction of the affected IgG with either naturally occurring or induced anti-GlcNAc antibodies; or third, through interaction of the affected IgG with antibodies induced against the peptide (or peptide-oligosaccharide) epitopes previously largely masked by the native oligosaccharide. Self-association of IgG could occur by any of these mechanisms (Fig. 8). Further, occupation of the vacant oligosaccharide-binding site by other serum or synovial fluid glycoprotein or cartilage components can also be envisaged.
Cellular Receptors to GlcNAc

IgG molecules presenting an abnormally high concentration of exposed non-reducing terminal GlcNAc residues may provoke immunopathological changes as a consequence of interaction with cells bearing receptors for the GlcNAc epitope [18]. Such receptors may be functionally important in the recognition of peptidoglycan structures of bacterial cell walls [37], in tumor surveillance by natural killer (NK) cells [1], and in macrophage recognition of cells undergoing programmed cell-death (apoptosis) [13]. Further, the membrane CR3 receptor which is expressed on phagocytic and NK cells, has been shown to bind GlcNAc-containing glycans [52]. This receptor also binds endotoxin, possibly via the N-acylated glucosamine-containing (lipid x) moiety [62]. In addition, certain macrophage functions have recently been shown to be activated by N-acetyl-chito-oligosaccharides (GlyNAc homopolymers) [55].

Antibodies to cross-reactive Glc-NAc-containing epitopes present in the environment, particularly the adjuvant bacterial components (GlcNAc containing peptidoglycans), would have interesting auto-immune effects. For example, immunisation of rabbits with *Mycobacterium smegmatis* results in antisera which recognise highly branched N-linked oligosaccharides terminating in N-acetylglucosamine residues and containing a bisecting GlcNAc residue [8]. These anti-GlcNAc antibodies have been shown to bind to a cell-surface antigen associated with intrathymic and intraabursal maturation of chicken lymphocytes [7]. Moreover, patients with tuberculosis have varied levels of antibody to GlcNAc [4].
Antibody to terminal GlcNAc may also have some of the properties of rheumatoid factor (see above). It has recently been reported that mice immunised with the peptidoglycan/polysaccharide complex of Group A streptococci can be used as a source for production of monoclonal antibodies which bind to the terminal GlcNAc residues situated in the Cgt2 domain of serum IgG isolated from patients with rheumatoid arthritis or tuberculosis [51]. This study is important, since Group A streptococci, which are associated with rheumatic fever, appear to be able to evoke formation of antibodies which will bind to an epitope on the agalactosyl IgG present in rheumatoid arthritis. The Group A streptococci polysaccharide/peptidoglycan complex is rich in GlcNAc and patients with rheumatic fever are known to have raised levels of antibody to GlcNAc [33], and patients with rheumatoid arthritis have also recently been shown to have raised levels of antibodies which bind to the Group A streptococci [27].

Rheumatoid Arthritis and Tuberculosis

The presence of agalactosyl IgG in patients with tuberculosis is consistent with the suggestion that mycobacteria, or auto-antigens which cross-react with them might be aetiological agents in rheumatoid arthritis [58]. Immune-complexes containing self-associated IgG have been identified in the serum of tuberculosis patients [25], and clearance studies have shown that IgG from these patients behaves similarly to the 'aged IgG' present in rheumatoid arthritis serum [60]. In addition, several studies have demonstrated that the serum of patients with tuberculosis contains rheumatoid factor which may be of the IgG and IgA isotypes as well as the classical IgM antibodies [26].

Linkages between HLA-DR phenotypes and the ability to respond to skin tests with mycobacterial antigens have also hinted at an association between rheumatoid arthritis and mycobacterial infections. Patients with rheumatoid arthritis respond relatively poorly to the common mycobacterial (group i) antigen, as do patients with leprosy or tuberculosis [3]. Skin-testing of 86 leprosy patients with four types of mycobacteria demonstrated that HLA-DR4 (a risk factor for rheumatoid arthritis) was associated with high responsiveness to antigens specific for M. tuberculosis [40]. Skin-testing of patients with rheumatoid arthritis also showed an increased responsiveness to tuberculin in those patients with the HLA-DR4 haplotype [3]. In contrast, rheumatoid arthritis patients with HLA-DR7 (a protective haplotype) showed low skin-test responsiveness to mycobacteria [3].

Further support for a connection between mycobacteria, or auto-antigens which cross-react with them, and rheumatoid arthritis comes from the finding of decreased levels of antibody binding to mycobacteria in those patients with rheumatoid arthritis who carry the relatively protective DR2 or DR7 haplotype [3, 4]. This class II MHC-associated regulation of antibody levels is an isotype-specific effect resulting in reduced IgM binding to mycobacteria in those patients with rheumatoid arthritis who are HLA-DR7, and reduced IgA binding to mycobacteria in those patients who are HLA-DR2. More recently, two groups (G. M. Bahr, personal communication; G. Tsoulfa, personal communication) have found preliminary evidence that patients with rheumatoid arthritis, have increased levels...
of antibody against a 65-kDa protein from *M. tuberculosis* [59] (supplied by Dr. Jan van Embden) relative to matched controls. There is also evidence that this particular mycobacterial antigen (i.e., 65-kDa protein) is relevant at the cellular level. A strongly arthritogenic T cell clone has recently been established from rats with an adjuvant-induced arthritis. This clone recognises both *M. tuberculosis*, and antigens present in human cartilage and synovial fluid [58]. More recently it has been shown that the relevant mycobacterial antigen is the 65-kDa protein discussed above, and that the peptide which it recognises shows some sequence homology with the link protein from cartilage.

Studies with T cells from patients with rheumatoid arthritis are at present incomplete. It is not yet clear whether the increased proliferative response of lymphocytes from such patients to an acetone-precipitated fraction of *M. tuberculosis* is related to the disease, or to the presence of an excess of DR4 individuals in the patient group [21]. If it was related to the disease itself, it will be important to know whether the observation was due to the presence of the 65-kDa protein in this preparation.

Finally, arthritic symptoms have been described in patients who were treated with *M. bovis* BCG immunotherapy for cancer [57]. These patients frequently experience early morning stiffness, finger and wrist swelling with signs of inflammation, and show a response to non-steroidal anti-inflammatory drugs. It is also interesting to note that the introduction of gold therapy for the treatment of rheumatoid arthritis followed the observation that the chronic disease process of tuberculosis resembled the chronic synovitis found in rheumatoid arthritis [14].

Differences in pathology between rheumatoid arthritis and tuberculosis (or Crohn's disease) secondary to the production in each of IgG with agalactosyl Fc may arise either through differences in the anatomical sites of production of such IgG, or through additional differences in Fab N-glycosylation. For example, the site of production of IgG with agalactosyl Fc (i.e., intra-articular or otherwise) would be expected to affect both the concentration-dependent self-association of such IgG (see earlier) as well as the clearance of the resulting complexes. Tentative schemes for the origin and consequences of increased IgG(0) in rheumatoid arthritis and tuberculosis are shown in Fig. 9.

**Mycobacteria and Crohn's Disease**

The presence of the agalactosyl IgG in Crohn's disease may also be a clue to its aetiology. For many years Crohn's disease was not considered to be distinct from intestinal tuberculosis, since the granulomas seen in Crohn's disease are similar in histology to tuberculoid granulomas. In 1932 Crohn, Oppenheimer, and Ginsberg defined "regional ileitis" as a distinct clinical and pathological entity but they did not rule out a role for mycobacteria in its pathogenesis [11]. It is now recognised that Crohn's disease can affect any part of the gastrointestinal tract. Food allergy, autoimmunity and infection due to an unknown transmissible agent, which could be an unusual type of mycobacterium, have been suggested as the cause. Homogenates of Crohn's disease tissue have been shown to induce unusual tissue reactions and granuloma formation in the guts of rabbits [6, 53] and in the
Fig. 9a, b. Tentative models for the production of IgG with agalactosyl Fc [IgG(0)] in response to external GlcNAc epitopes in rheumatoid arthritis (a) and tuberculosis (b). It is suggested that the decreased activity of β-galactosyltransferase in B lymphocytes is a response to lymphokines released by T lymphocytes. The anatomical site of production of IgG(0) may influence the subsequent pathological damage caused by IgG (0), and may account for differences between diseases that are associated with increased IgG (0). IL-1: Interleukin 1; TNF: tumour necrosis factor; I.C.: immune-complexes.

Footnotes:

Slow growing cell-wall defective organisms have been isolated from mesenteric lymph nodes draining the lesions of 22 out of 27 patients with Crohn’s disease, and only 1 of 11 controls [5]. However, the only evidence that these are unusual mycobacteria is their positive staining by the Ziehl-Neelsen technique. Recent research has confirmed the presence of such organisms in tissue from inflammatory bowel disease, but not from normal tissue; unfortunately these organisms could not be grown in sufficient quantity for further characterisation, although several groups have cultured an assortment of conventional mycobacterial species from Crohn’s disease tissue [5, 9, 17]. However, various mycobacterial species are common in biopsies of normal bowel wall [17] and there is no justification for linking these with Crohn’s disease. Present attention remains focused on the cell wall defective forms, which have the added attraction of being potentially filterable like the granulomagenic entity which was passaged in mouse footpads [35].
Agalactosyl IgG, Mycobacteria, and Tumour Necrosis Factor (TNF) Release

Agalactosyl IgG may be a regulator of TNF release from activated macrophages (G. Rook, unpublished). While this may be a consequence of agalactosyl IgG bound to the macrophage Fc receptor also binding via its exposed non-reducing terminal GlcNAc residues, either to some macrophage GlcNAc-binding receptor or to the GlcNAc-binding CR3 receptor, it is possible that agalactosyl oligosaccharides confer a sufficient affinity for the carbohydrate-dependent interaction between IgG and the Fc receptor to cause direct activation of macrophages and subsequent TNF release [32]. High release of TNF could indeed account for several clinical features of disease states which are characterised by IgG molecules with exposed non-reducing terminal \(N\)-acetylglucosamine residues. It has been demonstrated that live virulent \(M.\) \(tuberculosis\) can substitute for endotoxin in triggering TNF release and much of the necrosis in tuberculosis can be attributed to endothelial cell damage and microcapillary thrombi [50]. TNF is readily detected in the synovial fluid of patients with rheumatoid arthritis [22]. Further, established synovitis in patients with rheumatoid arthritis is associated with the proliferation of small blood vessels. Recent data have demonstrated that in vivo TNF stimulates neovascularisation which is accompanied by a leukocyte infiltration (i.e., inflammation) [15]. Other well-established angiogenesis factors induce capillary vessel formation in the absence of an inflammatory response. It is interesting to note that chronic inflammatory diseases not associated with raised agalactosyl IgG levels (i.e., sarcoidosis, leprosy) are characterised by granulomas and massive macrophage activation, but with limited weight loss and fever, or necrosis, suggesting the absence of TNF release.

Conclusions

In conclusion, there is a shift in the population of IgG glycoforms towards those with a higher content of agalactosyl biantennary \(N\)-linked oligosaccharides in active rheumatoid arthritis (both juvenile and adult), tuberculosis, and Crohn's disease, but not in a variety of other rheumatological, inflammatory, or infectious conditions. This shift may contribute to disease pathogenesis both through immune-complex formation and through disturbance of a cellular network directed against the non-reducing terminal GlcNAc epitope. The precise pathology would in each case be modulated by the anatomical site(s) of production of such IgG, and also by the precise mechanism inducing this change in IgG glycosylation. Important amongst such mechanisms may be cross-reactivity between environmental and endogenous carbohydrate epitopes. It will be interesting to see if future research supports the idea that groups of diseases (e.g., rheumatoid arthritis, tuberculosis, Crohn's) are indeed related by a common aetiopathogenesis (i.e., \(G(0)\)).

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The Role of Antigen in Autoimmune Responses with Special Reference to Changes in Carbohydrate Structure of IgG in Rheumatoid Arthritis

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Evidence indicating an important role for antigen in the provocation of autoimmune responses is presented. Attention is especially focused on carbohydrate abnormalities in IgG in rheumatoid arthritis, since auto-sensitization to this molecule is thought to be of central importance in the pathogenesis of this disease.

A higher percentage of Fcγ oligosaccharide chains in the serum IgG of patients with rheumatoid arthritis lack terminal galactose residues relative to age-matched controls. This does not appear to be a characteristic feature of chronic inflammatory diseases in general. A new, more rapid assay for agalactosyl chains is described and shown to give results comparable to the more conventional biochemical analysis. The defect probably arises from a reduction in activity of B-cell galactosyltransferases. The galactose changes may contribute to the autoantigenicity of IgG and could facilitate the self-association of IgG rheumatoid factors.

Introduction

For many years, studies on the mechanisms underlying the development of autoimmune diseases were directed largely at the identification of abnormalities in the immune response. Recently, however, there has been a greater awareness of the possible role played by antigen and its presentation (see for example Roitt, [1]).
Thus, the importance of thyroid antigen in the induction of the autoantithyroglobulin response in obese strain chickens was shown clearly by the effects of neonatal thyroidectomy [2]. We have also reported on the relevance of epitopes dependent upon iodination of thyroglobulin in creating pathogenetic T‐cell effectors [3]. Furthermore, it is difficult to avoid linking the particular clusters of antibodies associated with specific diseases (e.g. anti-DNA and Sm in SLE, anti-SS-A and SS-B with Sjögren’s syndrome and so on) with some antigen‐directed process rather than simply a non‐antigen‐specific polyclonal activation mechanism. Although a variety of IgM autoantibodies appear to be produced spontaneously in normal individuals [4], it is unlikely that the high affinity IgG autoantibodies characteristic of most autoimmune disorders could be generated without an antigen‐driven response involving somatic mutation and selection.

The other area of research in this field which has burgeoned over the last few years concerns the role of Class II MHC expression. The nature of T‐cell recognition implies that potential autoantigens on the surface of cells which do not express Class II will be unable to stimulate the corresponding autoreactive T cells. Thus, the finding that thyroid cells from patients with thyrotoxicosis ‘inappropriately’ expressed Class II molecules on their surface [5] was most intriguing and stimulated a great deal of interest. It was shown subsequently that the T cell lymphokine γ-interferon could induce Class II expression on normal thyroid cells [6] and on the pancreatic β‐cells of diabetes‐prone but not the diabetes‐resistant strain of BB rat [7]. These findings raise the still unresolved question of whether Class II expression is a prerequisite for the induction of autoimmunity or is restricted to a role in maintaining the pathogenetic effect of the autoimmune response.

The present paper focuses on abnormal features of the antigen generally recognized to be of central importance in provoking the immunological reactivity ultimately responsible for joint erosion in rheumatoid arthritis, namely IgG itself.

**Serum IgG in patients with rheumatoid arthritis (RA) shows defective N‐glycosylation**

The oligosaccharides in the Fc region of IgG are N‐linked and predominantly have the structures shown in Figure 1, each sugar having either two terminal galactose residues [G(2)], one galactose and one N‐acetylglucosamine (GlcNAc) [G(1)], or two terminal GlcNAcs [G(0)] (Figure 1). The two oligosaccharides, one originating from each Cγ2 domain, form a bridge to hold the Cγ2 domains apart as shown in Figure 2, while the terminal galactose residues on the α (1–6) arms sit in a pocket on the surface of each domain. In 1985 [8] the striking finding was reported that the oligosaccharides in the IgG from patients with RA were deficient in galactose, with an increase in structures of the G(0) type. Later studies with a further group of patients, confirmed these findings, taking into account the natural change in IgG galactosylation with age [9]. It was also found that a similar defect occurred in the IgG of patients with juvenile RA so strengthening the view [10] that both juvenile and adult forms shared common pathogenetic mechanisms. Within the rheumatological disorders, the defect showed quite considerable disease specificity. For example, primary systemic lupus erythematosus (SLE), primary Sjögren’s disease, ankylosing spondylitis and polymyositis, had normally galactosylated IgG oligosaccharide structures. Only in
Role of antigen in autoimmune responses

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**Figure 1.** The predominant IgG Fc oligosaccharides. Gal, galactose; GlcNAc, N-acetylglucosamine; Man, mannose. Terminal galactose residues bind the lectin ricin and N-acetylglucosamine the monoclonal antibody or the lectin bandeiraea.

**Figure 2.** IgG structures. The second heavy chain domains in IgG (Cy2) are kept apart by the two N-linked biantennary sugars. One of the α (1-3) arms must terminate in an N-acetylglucosamine to form a bridge (arrowed) with the oligosaccharide on the opposing Cy2 domain. The terminal galactose residues on the α (1-6) arms fit snugly into pockets on the protein domains as shown. ○, Mannose; ◊, N-acetylglucosamine; ●, Galactose.

the case of secondary Sjögren's disease with SLE was defective galactosylation similar to that in RA observed.

Of the chronic inflammatory disorders studied, the majority of chronic infections, such as leprosy and klebsiella were normal. Quite surprisingly, however, patients with pulmonary tuberculosis (TB) had galactose defects similar to those seen in RA.

*A new assay for agalactosyl-IgG*

Since the strict biochemical analysis of the structure of the IgG sugars is time-consuming and sophisticated, we have developed a new, more simplified assay which gives an approximate measure of the degree of galactosylation. Purified IgG is dot-blotted onto nitrocellulose and on separate aliquots of the same sample, probes for terminal galactose and terminal GlcNAc are applied. Biotinylated ricin is used to
detect galactose, and either biotinylated bandeiraea lectin or monoclonal anti-GlcNAc are employed for the detection of terminal N-acetylglucosamine. The blots are developed by addition of streptavidin horseradish peroxidase conjugates and the colour reaction formed on the nitrocellulose. The colour intensity is recorded by transmitted colorimetry, and the ratio of the two stains gives a measure of the galactose: N-acetylglucosamine ratio. This ratio correlates closely with the percentage of galactose-free chains \([G(0)]\) determined by conventional biochemical techniques (Figure 3).

This faster assay gives comparable results, as may be seen from measurements made on the serum IgG from patients with RA (Figure 4). The majority of the IgG galactose-free values lie above the mean ± 2 standard deviations for the age-matched controls. The few sera which lie within the normal range are from patients with inactive disease. The elevated incidence of \([G(0)]\) in patients with pulmonary TB is confirmed and may be clearly seen in Figure 5.

Preliminary studies on the families of patients with RA indicated a clustering of relatives with the galactose defect in two out of seven such families. In one of these cases the unrelated spouse had an elevated galactose-free value. Further studies clearly are required to see whether this finding can be substantiated.

**Implications of this structural galactose defect**

The ability of IgG rheumatoid factors to form self-associated complexes capable of stimulating the chronic inflammatory change giving rise to the erosive pannus in RA,
has long been recognized. While it has been assumed generally that the binding between the Fab on one rheumatoid factor molecule, and the Fc of another, was due to conventional antigen/antibody links based upon the hypervariable region of the combining site, the present studies suggest a further possible mechanism by which the self-association could be strengthened. Since it has been established that the Fab oligosaccharides which occur on approximately one in three different immunoglobulin molecules, are not defective with respect to glycosylation in RA, a Fab galactose region could become inserted in the Fc pocket left vacant by a galactose-deficient Cy2 oligosaccharide (Figure 6).

This suggests a new possibility for therapeutic intervention, since if the carbohydrate moieties contribute significantly to the formation of the self-associated complex, then it might be possible using the appropriate sugars, to interfere with aggregate formation. Early experiments have been encouraging in this respect.

The defect may arise from low activity of galactosyltransferase

In order to examine the possibility that the change in N-glycosylation of serum IgG in RA was the result of impaired activity of the galactosyltransferase enzyme responsible for addition of the galactose residue to terminal N-acetylgalactosamine, we measured the activity of this enzyme in peripheral B- and T-lymphocytes of patients with RA, using ovalbumin as the receptor glycoprotein [11]. Enzyme activity in B

Figure 4. Analysis of RA using new assay. Galactose defect in IgG patients with rheumatoid arthritis analysed by the dot blot assay. The percentage G(0) values are plotted against age. The outer bounds of the hatched lines depict the regression functions for ±2 (SD) of the mean. •, controls; O, rheumatoid patients.
Figure 5. Analysis of pulmonary tuberculosis using new assay. Galactose defect in IgG patients with tuberculosis analysed by the dot blot assay. The percentage \( G(0) \) values are plotted against age. The outer bounds of the hatched lines depict the regression functions for \( \pm 2 \) (SD) of the mean \( \bullet \), controls; \( \circ \), patients with tuberculosis.

Figure 6. Self-association of IgG rheumatoid factor. (a) Ig rheumatoid factor; (b) Self-associated IgG rheumatoid factor.

cells from patients was considerably lower than that observed in control subjects using ovalbumin and a variety of other high molecular weight glycoprotein receptors as well as GlcNAc itself. A lesser but still significant diminution in activity was seen in the T cells (Figure 7). We have mentioned earlier that the IgG from untreated pulmonary TB subjects had raised \( G(0) \) levels, and it was of considerable interest that both B- and T-lymphocytes from the peripheral blood of such patients showed
grossly lowered galactosyltransferase activity. It has been postulated that Crohn's disease is mediated by some abnormal type of mycobacteria, and it may thus be relevant to note that in the patients with active as distinct from inactive Crohn's disease, serum IgG shows a raised G(0), and peripheral blood B cells and T cells a lowered galactosyltransferase.

Conclusions

Similarities in galactose deficiency in the IgG from patients with RA and primary TB, point rather tantalizingly to some possible connection between an infective agent and RA. Even more provocative in this respect, is the production of adjuvant arthritis in rats by T-cell clones sensitized to the 65 kD protein of *Mycobacterium tuberculosis*. This molecule belongs to the group of substances collectively termed 'heat shock proteins'. Should injection of such clones induce changes in the galactosylation of serum IgG, one's interest must turn to the possibility that interaction of homologous human heat shock proteins with the immune system (perhaps as a result of a cross-reaction induced by sensitization with some microbial product), might trigger the IgG galactose defect so leading to IgG autosensitization and possibly the train of pathogenic events which lead finally to joint erosion. Recent results by Peter Lydyard and his colleagues (Tsoulfa *et al.* in preparation) confirmed by a study of a further group of patients in Kuwait (Barr *et al.* in preparation) suggest that patients
with RA have abnormally high levels of IgG and IgA antibodies to heat shock proteins of human as well as mycobacterial origin. These new insights into the puzzling nature of RA should provide exciting prospects for future study.

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