

Agalactosyl IgG: its clinical associations, biological properties, and
relationship to infection

**Agalactosyl IgG:
its disease association and biological properties**

by

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MY FAMILY

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Abbreviations

PBS--phosphate buffered saline
PBS/T20--phosphate buffered saline/Tween 20
ABTS--2,2'-Azino-di-(3-ethyl benzithiozoline sulfuric acid)
CPB--citrate phosphate buffer
G(0)--agalactosyl IgG
BSA--bovine serum albumin
FCS--foetal calf serum
FBS--foetal bovine serum
ELISA--enzyme linked immunosorbent assay
GlcNAc--N-acetylglucosamine
ER--endoplasmic reticulum
Man--mannose
ADCC--antibody dependent cell-mediated cytotoxicity
RA--rheumatoid arthritis
IL-6--interleukin 6
RF--rheumatoid factor
MHC--major histocompatibility complex
hsp--heat shock protein
TB--tuberculosis
CD--Crohn's disease
TNF--tumour necrosis factor
SDS--sodium dodecyl sulphate
PAGE--polyacrylamide gel electrophoresis
DMSO--dimethyl sulfoxide
PPD--purified protein derivative of *Mycobacterium tuberculosis*
MTT--3-(4-5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide
CRP--C-reactive protein
TGF- β --transforming growth factor
EDTA--ethyl disodium tetra
FITC--fluorescein isothiocyanate
DNP--dinitrophenol
TNP--trinitrophenol
SRBC--sheep red blood cells
PFC--plaque forming cells
RhD--Rhesus D antigen
CFA--complete Freund adjuvant

SUMMARY

In this study, the immunoassay for measuring the level of agalactosyl IgG has been developed into a more simple and efficient version. Using this method, an increase in serum level of agalactosyl IgG has been demonstrated in patients with rheumatoid arthritis, sarcoidosis and Takayasu's arteritis.

The second part of the study addressed the regulation of agalactosyl IgG by interleukin-6 (IL-6). A significant correlation has been shown between serum IL-6 titres and percentage agalactosyl IgG in patients with sarcoidosis. The serum level of agalactosyl IgG was also correlated with the IL-6 titres in peritoneal exudate fluid in pristane-injected mice. Moreover, the injection of recombinant IL-6 into normal mice increased their serum level of agalactosyl IgG. However, in patients with rheumatoid arthritis, no significant correlation between agalactosyl IgG and IL-6 was found. Furthermore, an inhibitor of IL-6 previously found in sera of sarcoidosis patients using the B9 cell line was shown not to be specific for IL-6, and inhibited the growth of other cell types.

In the third part of the study, a reduction was demonstrated in binding of the agalactosyl IgG3 to human Fc γ RII isoforms compared to the galactosyl IgG3. This suggested the possibility that negative feedback on B cells via Fc receptors might be defective, but *in vivo* experiment with agalactosyl IgG showed that such feedback was normal. The importance of these findings was discussed in relation to autoimmune diseases and abnormal humoral immune response evoked by infectious agents.

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Chapter 1.

General Introduction

1. 1. IgG and its glycosylation

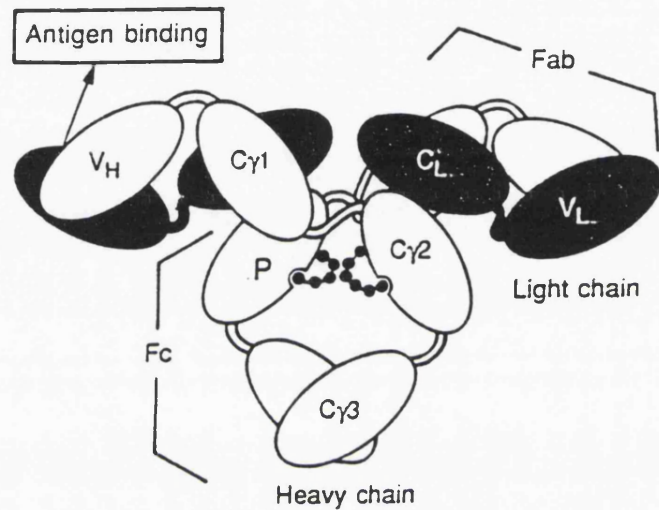
1.1.1. The structure of IgG

The immunoglobulins (Igs), or antibodies, are a group of glycoproteins present in the serum and tissue fluids of all mammals. They are produced by plasma cells which are derived from B lymphocytes. There are five distinct classes of immunoglobulin molecules recognised in most higher mammals, namely IgG, IgA, IgM, IgD and IgE. They differ from each other in size, charge, amino acid composition and carbohydrate content.

IgG is the most abundant antibody class in normal human serum accounting for 70-75% of the total immunoglobulin pool. An IgG molecule has four polypeptide chains, consisting of two identical light (L) chains (which may be of either the κ or λ isotypes) and two identical heavy (H; γ) chains (which may be of one of four subclasses), linked together by interchain disulfide bonds and non-covalent forces in the form L-H-H-L (Fig. 1.1).

Internal sequence homologies provided the first indication that the L and H polypeptide chains are folded into discrete domains each consisting of about 110 amino acids and containing a 60 residue disulfide loop. This has been confirmed by electron microscopy and

Figure 1.1 The domain structure of IgG



This diagram is modified from "Essential Immunology" (Roitt, 1991). The two oligosaccharides (•••••) attached at Asn-297 hold apart the two $C\gamma2$ domains. "P" represents a lectin-like pocket on the surface of the $C\gamma2$ domain.

X-ray crystallographic analysis. The IgG L chains are folded into two domains while the H chains are composed of four domains (Fig. 1.1). The amino-terminal end of both the L and H chains show extensive sequence variation associated with their antigen-binding specificity, and these globular regions are therefore referred to as variable domains (V_H and V_L). Whereas the carboxyl-terminal end of both the heavy and light chains have a relatively constant structure and only exhibit subclass (isotype) and allotypic variation in amino acid sequence. There are four subclasses of the γ chains and there are allotypic variations in these subclasses. The constant (C) portion of IgG light chain is termed the C_L domain. The constant portion of the γ chain is further divided into three domains: $C_{\gamma 1}$, $C_{\gamma 2}$ and $C_{\gamma 3}$. The mid-section of the γ chains, between the $C_{\gamma 1}$ and $C_{\gamma 2}$ domains, is characterised by a region of extended polypeptide chain known as the hinge region. Flexibility in this area allows the two antigen-binding sites to operate independently and engage bivalently with randomly spaced epitopes. Moreover, there is close pairing of the domains except in the $C_{\gamma 2}$ domains which are separated by the carbohydrate moieties (Fig. 1.1).

Another feature about the hinge region is the proteolytic susceptibility. For example, papain can readily cleave the IgG molecule at the hinge region, and dividing the molecule into two functional units -- a pair of Fab (fragment antigen-binding) fragments, each with one antigen-binding site; and a single Fc (fragment crystallizable) fragment, which readily crystallises (Fig. 1.1). The Fc region is particularly important in determining the effector functions of IgG. Another protease, pepsin, on the other hand, can produce one

F(ab')₂ fragment, which is essentially two Fab fragments still linked together by disulfide bonds and remain bivalent in antigen-binding; and the rest of the molecule is degraded into smaller fragments. Fragments corresponding to individual intact domains appear to retain their original folding and continue to exhibit interdomain association as well as some of the biological properties associated with that domain.

1.1.2. The functions of IgG

Like all other classes of antibody, the essential role of secreted IgG antibody is to act as a bifunctional molecule (adapter) which can bind to antigen via specific antigen-binding sites and then crosslink the antigen to cells of the immune system or activate the complement system. Antigen-binding is the primary function of the Fab region of IgG. Within the variable domains, there are three short polypeptide segments which are found to express exceptional sequence variability and these segments are termed hypervariable regions. In both heavy and light chains, such hypervariable regions are located approximately between amino acid residues 30-36, 50-65 and 93-102. Although separated in the primary sequence, these hypervariable regions are brought together by the folding of the variable domains and appear as loops at the tip of the immunoglobulin molecule. The association of the variable domains of a heavy and a light chain therefore generates a surface with six exposed hypervariable loops, three from each chain. This surface forms the antigen binding site and these hypervariable regions are sometimes also called the complementarity determining

regions (CDRs). The role of the C γ 1 and C $_L$ domains is to secure the interchain binding to the L chain to provide a base for the antigen-binding variable domains.

The Fc region of IgG is the locus responsible for the effector functions of the molecule. The principal effector functions triggered by IgG are complement activation, cellular elimination mechanisms such as phagocytosis and antibody-dependent cellular cytotoxicity (ADCC), and immune regulation by feedback control on IgG antibody synthesis.

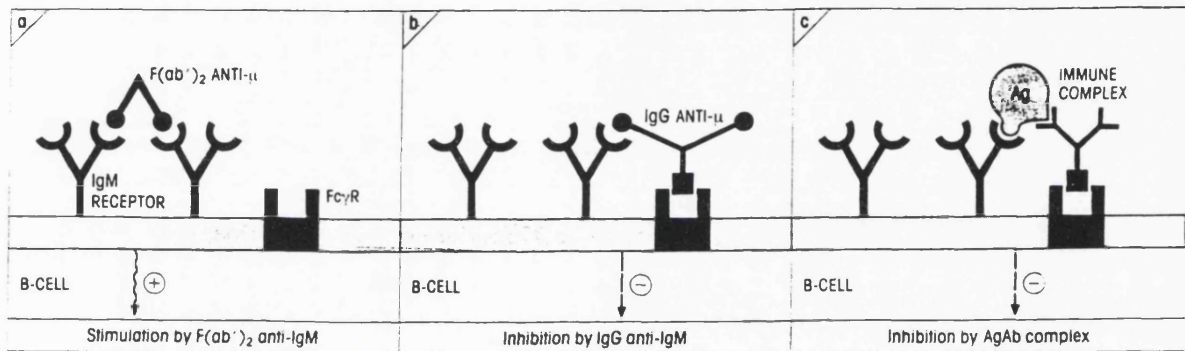
It is the classical pathway of complement activation where IgG is involved. The classical pathway of complement is triggered by the interaction of the first complement component, C1, either with IgG in an associated state (e.g. coating a target cell or aggregated by antigen in an immune complex) or with IgM bound to antigen. C1 is a complex consisting of subcomponents C1q, C1r and C1s. C1q interacts with antibody to initiate the enzymatic process of the pathway. The site where C1q binds IgG has been located on the C γ 2 domain of the Fc fragment.

Besides activating the complement system, the Fc region of an IgG molecule can bind to specific Fc receptors (Fc γ R) expressed on many types of cells and mediate a variety of effects. For example, a bacterium coated with specific IgG antibodies can be efficiently phagocytosed by macrophages or neutrophils through the binding of Fc region of IgG antibodies to the Fc γ R on these phagocytes. Various types of white blood cells that express Fc γ R can also kill IgG-coated foreign eukaryotic cells without phagocytosing them. This process, called ADCC, can be carried out by macrophage, neutrophils, as well

as natural killer cells (NK cells). In addition, Fc γ R are also expressed on the membrane of B lymphocytes. It has been well established that normal IgG bound to Fc γ R on B-cell membranes can exert negative feedback control on antibody production by the B cell if it is cross-linked with the membrane immunoglobulin (mIg) of the same cell (Phillips and Parker, 1984; Sidman and Unanue, 1976; Rudich *et al*, 1985; Waldschmidt *et al*, 1983; Bijsterbosch and Klaus, 1985; Sinclair, 1991). This can occur when the IgG is an anti-mIg, or an anti-idiotypic, or in the presence of a multivalent antigen to which both the mIg and the Fc-bound IgG can bind (Fig. 1.2). On the other hand, IgG molecules are the only antibodies that can pass from mother to foetus via the placenta. Cells of the placenta that are in contact with maternal blood have Fc receptors that bind IgG molecules and mediate their passage to the foetus. The IgG antibodies are first ingested by receptor-mediated endocytosis and then transported across the cell in vesicles and released by exocytosis into the foetal blood (a process called transcytosis). Other classes of antibodies do not bind to these receptors and therefore cannot pass across the placenta.

Binding of IgG also occurs to some bacterial cell wall proteins such as protein A of *Staphylococcus aureus* and protein G of *Streptococcus* strains C and G (Goding, 1978). The sites for the interaction between protein A and IgG has been shown by X-ray crystallography to extend over the C γ 2 and C γ 3 domains. Over the years, protein A has been proved to be an extremely versatile reagent for isolation and detection of IgG, because of its selective high affinity binding to IgG. Protein G has been found to have different affinities for subclasses and species of IgG compared to protein A (Goding,

Figure 1.2 Negative feedback control on antibody synthesis



This diagram (Roitt, 1991) illustrates that cross-linking of Fc γ R and surface IgM (μ heavy chain) on a B cell by intact IgG anti- μ (b) or by antigen-antibody (AgAb) immune complex (c) leads to inhibition of B-cell function.

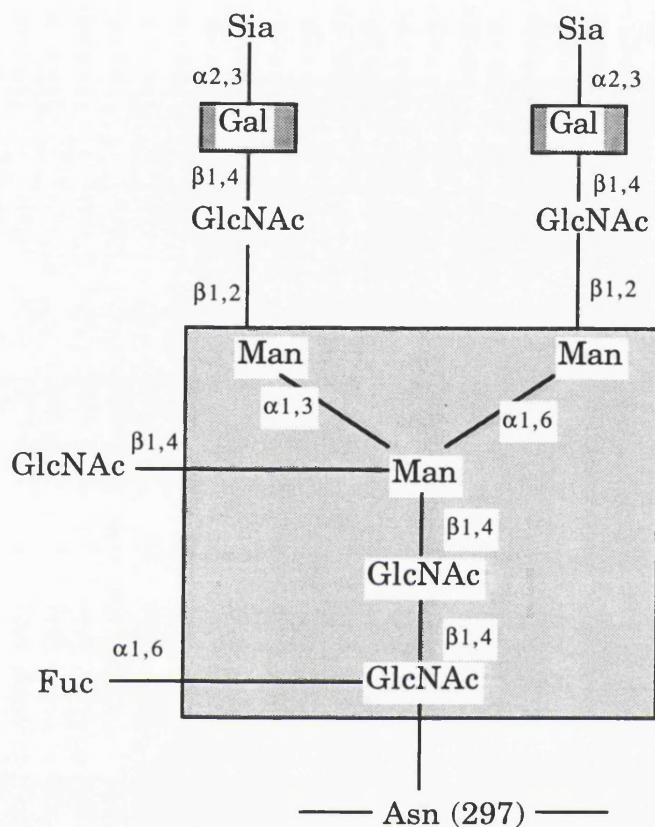
1978; Langone, 1982). Therefore, some subclasses and species of IgG can only bind very weakly to protein A, and in this case protein G may be used instead to obtain much stronger affinity binding.

1.1.3. The process of protein *N*-glycosylation

Many secreted and membrane-associated proteins including antibodies contain covalently attached oligosaccharide units. These oligosaccharides are attached to either the side chain oxygen atom of serine (Ser) or threonine (Thr) residues by *O*-glycosidic linkages or to the side chain nitrogen of asparagine (Asn) residues by *N*-glycosidic linkages. The *N*-linked oligosaccharides appear to be the most common ones found in glycoproteins. These *N*-linked oligosaccharides contain a common core region consisting of three mannose and two *N*-acetylglucosamine (GlcNAc) residues (Fig. 1.3). Additional sugars are attached to this common core in many different ways to form a variety of oligosaccharide patterns found in glycoproteins.

Glycoproteins acquire the core sugars of the *N*-linked oligosaccharides in the endoplasmic reticulum (ER). A somewhat larger precursor oligosaccharide block consisting of fourteen sugar residues (two GlcNAc, nine mannoses and three glucose) is firstly constructed on an activated lipid carrier, dolichol phosphate, by sequential addition of single sugar residues. The block is then transferred from this highly hydrophobic carrier to an asparagine side chain of a nascent protein in the lumen of the ER. Three glucose residues and a mannose residue are rapidly trimmed while the

Figure 1.3 The general structure of *N*-linked complex-type oligosaccharide



The *N*-linked complex-type oligosaccharide consists of a core region (shaded part) and a terminal region. The oligosaccharides attached at Asn-297 in the C γ 2 domain of human serum IgG molecule are of bi-antennary complexed-type, which are usually asialyated and lack terminal galactose on the α 1-3 arm of at least one oligosaccharide chain (Parekh *et al*, 1985).

Fuc : Fucose, Gal : Galactose, GlcNAc: N-Acetylglucosamine,
 Man : Mannose, Sia : Sialic acid, Asn : asparagine.

glycoprotein is still in the ER. Transfer vesicles then carry the protein from the ER to the Golgi apparatus for further modifications of the oligosaccharides.

Two broad classes of *N*-linked oligosaccharides, the complex-type oligosaccharides and the high-mannose oligosaccharides, are found in mature glycoproteins. Sometimes both types are attached (in different places) to the same polypeptide chain. High-mannose oligosaccharides have no new sugars added to them in the Golgi apparatus. They contain just two GlcNAc residues and many mannose residues. Complex-type oligosaccharides, by contrast, can contain more than the original two GlcNAc residues as well as a variable number of galactose and sialic acid residues and, in some cases, fucose (Fig. 1.3).

The complex-type oligosaccharides are generated by a combination of further trimming of the original oligosaccharides added in the ER and the addition of further sugars. Thus each complex-type oligosaccharide consists of a core region, derived from the original *N*-linked oligosaccharide and typically containing two GlcNAc and three mannose residues, plus a terminal region consisting of a variable number of GlcNAc-galactose-sialic acid trisaccharide units linked to the core mannose residues. Frequently the terminal region is truncated, containing only GlcNAc and galactose, or even just GlcNAc. In addition, a fucose residue may or may not be added, usually to the core GlcNAc residue attached to the asparagine. All the terminal-region sugars are added *intrans* in the Golgi by a series of glycosyl transferases (GlcNAc transferase II, GlcNAc transferase IV, galactosyltransferase, and sialyltransferase) that act in a rigidly

determined sequence.

1.1.4. Glycosylation of IgG

All normal serum immunoglobulins carry *N*-linked oligosaccharides. These *N*-linked oligosaccharides are located at conserved *N*-glycosylation sites on the Fc, and at non-conserved sites on the Fab. In addition, immunoglobulins from certain species also carry *O*-linked oligosaccharides. For example, rabbit IgG carries one or two *O*-linked oligosaccharides in the hinge region (Taniguchi *et al*, 1985), but human serum IgG invariably lacks *O*-linked oligosaccharides.

The detailed structural analysis of IgG glycosylation has revealed that human serum IgG carries, on average, 2.8 *N*-linked oligosaccharides (Parekh *et al*, 1985; Rademacher *et al*, 1986). Of these, 2.0 are invariably located in the C γ 2 domain at the conserved *N*-glycosylation site of Asn-297, and the remaining oligosaccharides are found attached to the variable regions of the light and heavy chains, with a frequency and location dependent on the occurrence of an Asn-X-Ser (Thr) *N*-glycosylation site (where X is any amino acid except proline) during immunoglobulin synthesis. There are 31 different complex-type oligosaccharides which have been found to associate with total human serum IgG. These structures are nonrandomly distributed between Fab and Fc regions. The Fc-associated oligosaccharides are characterised by a low incidence of sialylated structures and of 'bisecting' GlcNAc in the cores, and by the absence of galactose on the α 1-3 arm of at least one oligosaccharide chain in

the Fc region (Fig. 1.3). Those oligosaccharides on the Fab are characterised by a high incidence of sialylated structures, and carrying a 'bisecting' GlcNAc in the cores (Rademacher *et al*, 1986; Taniguchi *et al*, 1985). The large number of different oligosaccharides associated with IgG is not the result of analysis of a polyclonal population, since monoclonal antibodies in general also show a heterogeneity of glycosylation similar to that found in the total serum IgG of the corresponding species (Rademacher *et al*, 1986; Mizuochi *et al*, 1982). Further, the pattern of *N*-glycosylation of serum IgG is broadly similar between species. In general, the oligosaccharide structures on any IgG can be accommodated within the same set of the 31 oligosaccharides found on human IgG. Only the relative incidence of individual structures varies between species (Rademacher *et al*, 1986).

1.1.5. The biological significance of IgG oligosaccharides

The X-ray crystallographic analysis of the Fc fragment of rabbit IgG suggested an important role of the Fc-associated oligosaccharides in maintaining the three dimensional structure of the Fc region (Sutton and Phillips, 1983). The two oligosaccharide chains, each attached at Asn-297, are in direct contact with each other, and form a bridge across the two C γ 2 domains (Parekh *et al*, 1985). The α (1-3) arm of one oligosaccharide chain is always devoid of galactose and interacts through its β (1-2)GlcNAc residue with the Man β (1-4)GlcNAc segment of the opposing oligosaccharide chain (Fig. 1.3). While the α (1-3) arm of the opposing oligosaccharide chain extends outwards between the domains with no apparent steric constraints on its length.

The importance of the oligosaccharide chains for the biological function of IgG molecules has been suggested by the studies using aglycosylated IgG preparations. The aglycosylated IgG were either generated by treatments with various glycosidases to remove the oligosaccharides from IgG molecules, or produced by antibody-producing hybridoma cells in the presence tunicamycin, an antibiotic inhibitor of *N*-glycosylation (Koide *et al*, 1977; Nose and Wigzell, 1983; Leatherbarrow *et al*, 1985; Heyman *et al*, 1985; Walker *et al*, 1989). It was demonstrated that depletion of oligosaccharides from IgG molecules affected the ability of IgG to activate complement (C1q binding), to interact with FcγR, to induce ADCC, and to negatively feedback control antibody synthesis, but retained their capacity to bind antigens and protein A. These results revealed that alteration of the oligosaccharide chains of IgG molecules accompanies the reduction or disappearance of the functions carried by their Fc region. This may be due to the instability of the Cγ2 domains caused by the oligosaccharide modifications, since the aglycosylated IgG became more susceptible to pepsin or trypsin digestion (Tao and Morrison, 1989). However, this effect is not extended to the Fab region where antigens bind, nor to the interdomain region between Cγ2 and Cγ3 where protein A binds. Nevertheless, there have been suggestions that the Fab oligosaccharides may affect solubility or aggregation of IgG (Rademacher *et al*, 1988).

1.2. Agalactosyl IgG and its disease association

1.2.1. Definition of agalactosyl IgG

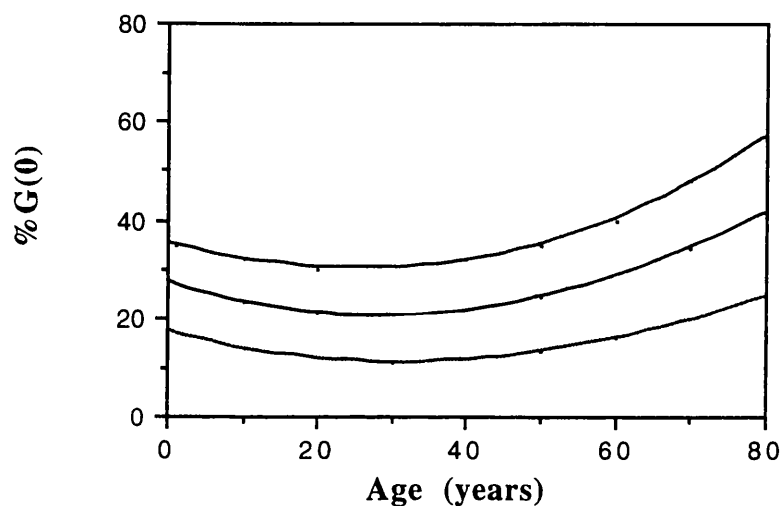
Agalactosyl IgG [G(0)] is defined as IgG molecules bearing two paired Fc-associated oligosaccharides both completely lacking galactose, terminating in GlcNAc (Rademacher *et al*, 1988). These oligosaccharide chains were referred to as G(0) (no galactose) in contrast to G(2), G(1), which are oligosaccharides having two or one terminal galactose, respectively. Since each IgG molecule contains two paired oligosaccharide chains, any combination of G(0), G(1) and G(2) are possible. Agalactosyl IgG contains G(0)-G(0) homologous pairing. The percentage of IgG bearing G(0) is referred to as %G(0).

Normal individuals have a substantial quantity of circulating agalactosyl IgG, which has been found to vary with age (Parekh *et al*, 1988a). The levels of agalactosyl IgG are about 30% in small children, and fall to around 20% by age of 25, and then steadily increase with age to 40% by the age of 70 (Fig. 1.4). It has also been found that the %G(0) falls during normal pregnancy, reaching a trough at delivery, and then rises sharply post parturition (Rook *et al*, 1991c).

1.2.2. Agalactosyl IgG and disease association

A decrease in IgG galactose content in RA was first reported in 1975 (Mullinax, 1975). Ten years later, in 1985, a study was carried out to compare the detailed structure of N-linked oligosaccharides of total serum IgG from normal individuals, with that from patients with

Figure 1.4 The change of %G(0) with age in normal individuals



This plot shows that agalactosyl IgG is age-related (Parekh *et al*, 1988). The middle line represents the mean percentage of agalactosyl IgG at various ages in normal individuals. The area in between of the top and the bottom lines indicates two standard deviations (2x SD) either side of that mean.

active RA, and primary osteoarthritis (Parekh *et al*, 1985). It was shown that patients with RA had a marked elevation of %G(0), and patients with osteoarthritis had a slight increase in %G(0), in comparison with normal individuals. It was subsequently revealed, in a study of 151 healthy individuals varying in age from 1 to 70 years, that the %G(0) was age-related and it was essential to relate the %G(0) value to a reference age curve (Fig. 1.4, Parekh *et al*, 1988a). When this was considered, the obtained %G(0) in patients with osteoarthritis was in fact within the normal range, but the %G(0) in patients with RA still remained well above the normal range (Parekh *et al*, 1988b). In addition, patients with juvenile onset rheumatoid arthritis were also found to have abnormally high %G(0). The raised %G(0) was correlated with disease activity in both adult and juvenile onset rheumatoid arthritis (Parekh *et al*, 1988b).

However, such an increase in %G(0) was not specific to RA, but also found in patients with tuberculosis and Crohn's disease. In contrast, the %G(0) was reported to be normal in other diseases including SLE (unless Sjogren's syndrome is also present), osteoarthritis, multiple sclerosis, sarcoidosis, leprosy, *Klebsiella* infections, myositis and a range of viral infections (Parekh *et al*, 1989). In this study, the %G(0) was analysed in samples from approximately 25 different diseases, using the accurate but very labour-intensive and time-consuming biochemical method which was described by Parekh *et al* (1985). Some of these diseases were therefore only tested with very small number of samples. To facilitate the analysis of agalactosyl IgG with significant number of samples in various diseases, two novel methods were subsequently developed,

one of which was an immunoassay using a monoclonal anti-GlcNAc antibody raised by immunizing mice with group A streptococci (Filley *et al*, 1989; Rook *et al*, 1988); the other was a lectin binding method using the differential binding properties of certain lectins to particular sugars (Sumar *et al*, 1990). Using the immunoassay, the increase in %G(0) was also demonstrated in a large number of serum samples from patients with Crohn's disease (Dube *et al*, 1990). In addition, a transient rise in %G(0) was found in leprosy patients during episodes of erythema nodosum leprosum (ENL) (Filley *et al*, 1989). There was no rise in %G(0) in patients with acute rheumatic fever (Bahr *et al*, 1990). The increase in %G(0) was also demonstrated in another set of patients with juvenile and adult onset rheumatoid arthritis using the lectin binding assay (Sumar *et al*, 1991). Moreover, it has been reported that the level of agalactosyl IgG may be used as an aid to differential diagnosis in early synovitis (Young *et al*, 1991). Thus, when patients presenting early synovitis had both raised %G(0) and positive rheumatoid factor, 94% of them developed RA two years later; when those patients with raised %G(0) only, 80% of them became arthritic. Further, the association between the levels of agalactosyl IgG and the disease activity has also been demonstrated during pregnancy of RA patients (Rook *et al*, 1991c). It has long been observed that patients with RA go into remission when they become pregnant. Serial bleeds from a normal pregnant woman showed a fall in %G(0) during gestation and a rapid rise post-partum. The same pattern of changes in %G(0) was also shown in an arthritic pregnant woman who had an abnormally elevated %G(0) before conception, and these changes occurred simultaneously with the pregnancy-

induced remission and post-partum relapse of the disease.

In addition to certain disease states in human, raised levels of agalactosyl IgG were also shown in mice with collagen-induced arthritis and pristane-induced arthritis (Rook *et al*, 1991c & 1991a).

1.2.3. Rheumatoid arthritis

1.2.3.1. The pathogenesis of rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease of the synovium which results in erosion, deformity and destruction of joints. The dominant pathological features of the lesion include: synovial cell proliferation, lymphoid cell infiltration and inflammation. Frequently, these features are found to co-exist in RA synovial specimens, thus it has been difficult to define the sequence of the events in the pathogenesis of RA. Nevertheless, it is generally believed that local immune responses play an important role, although the actual trigger of these responses leading to joint damage is unknown. Some immunologists have suggested the following sequence of events (Schwartz and Datta, 1989). Thus an unidentified agent or mechanism initiates an inflammatory response in the synovium, which results in the damage of small blood vessels, and accumulation of various types of cells. In the inflamed synovial membrane, CD4⁺ helper T cells predominate; plasma cells, macrophages, dendritic cells are also found. Immune complexes and numerous cytokines, such as interleukin-1 (IL-1), IL-6, tumour necrosis factor- α (TNF- α), granulocyte-macrophage colony-

stimulating factor (GM-CSF), can be detected in the synovial fluid. These cytokines are probably produced as a result of local T cell and macrophage activation. IL-1 and TNF- α activate resident synovial cells to produce hydrolytic enzymes, such as collagenases, that mediate destruction of the cartilage, ligaments and tendons of the joints. CD4⁺ T helper cells are activated by recognition of antigen-MHC class II complexes which are expressed on the surface of MHC class II positive cells, such as macrophages and dendritic cells. The activated T helper cells produce cytokines which recruit more CD4⁺ cells, B cells, polymorphs, and stimulate macrophages. At the same time, B cell growth factors, such as IL-6, cause the rapid maturation of B cells to plasma cells to secrete immunoglobulins. Some of these immunoglobulins are rheumatoid factors which bind to other IgG molecules in the joint to form immune complexes. These immune complexes, on the one hand, activate the complement cascade, leading to increased vascular permeability, infiltration with more inflammatory cells, and an influx of neutrophils. By this time, the joint is swollen, hot and painful. On the other hand, the immune complexes are phagocytosed by macrophages and polymorphs, which are then activated to release various lysosomal proteases and reactive oxygen intermediates. These soluble products damage collagen and the cartilage matrix, leading to erosion and destruction of the joint.

The concept that RA is an autoimmune disease is supported by the presence of various autoantibodies in RA patients' serum and synovial fluid. The most famous of these, discovered more than 50 years ago, is rheumatoid factor (RF), which are autoantibodies, usually of IgM and IgG classes, against the Fc region of IgG molecules.

However, RFs are not only present in RA, but also in other rheumatic disorders and some chronic infections (Carson *et al*, 1987). Moreover, transient RF production is also observed in healthy individuals after stimulation with polyclonal activators (Carson *et al*, 1991). Although not restricted to RA, rheumatoid factors, particularly in high titre, add weight to the diagnosis of RA. In addition to RFs, other autoantibodies, such as anti-collagen (Type II) antibodies, can also be found in RA. However, whether RA is really caused by these autoantibodies to the endogenous proteins, such as collagen and IgG, remain controversial, although collagen can cause experimental arthritis in rodents and higher animals (including monkeys), and collagen-induced arthritis is often considered to be a relevant model for RA (Stuart *et al*, 1984; Jasin, 1983). Most data from studies in human support the idea that these autoantibodies contribute to the aggravation of rheumatoid inflammation rather than initiation of the disease (Harris, 1990).

1.2.3.2. The aetiology of rheumatoid arthritis

The aetiological origins of RA are mysterious, but microbial, genetic, environmental and hormonal factors have been implicated. It is known that RA occurs predominantly in women. The disease susceptibility is associated with the human MHC (known as HLA, human leukocyte antigen) class II locus. A majority of patients with RA carry HLA-DR4, HLA-DR1, or both (McDermott *et al*, 1988). However, recent studies argued that HLA association is related to the severity of RA rather than the disease susceptibility (Silman, 1992).

The role of infectious agents in the aetiology of RA has long been considered an attractive possibility. Several organisms, including viruses (i.e. Epstein-Barr virus [EBV] and parvovirus), and bacteria (e.g. mycobacteria) have been implicated in causing RA (Harris, 1990; Moreland and Koopman, 1991). Interest in mycobacteria as potential aetiological agents of RA has been stimulated by observations that a mycobacterial 65-kD heat shock protein (hsp65) contains a nonapeptide sequence recognized by an arthritogenic T cell clone isolated from a rat immunized with killed mycobacteria in oil (van Eden *et al*, 1988). It is presumed that these T cells recognized a cross-reactive epitope expressed in normal joint tissue since transfer of the arthritogenic clone to unimmunized irradiated recipients induced arthritis (van Eden *et al*, 1985). A reasonable candidate in this regard is the recipient's homologous hsp, because hsps are highly conserved among bacterial and eukaryotic species (Jindal *et al*, 1989). However, it is not clear whether bacteria outside mycobacteria could similarly induce arthritis in this rat model, and whether the arthritogenic T cell clone isolated from the above described adjuvant arthritic rats could also recognize hsp65 homologues from mouse and other bacteria. In the case of human RA, synovial T cells were found to react with the mycobacterial hsp65 (Gaston *et al*, 1989). These T cell clones were subsequently shown to recognize an N-terminal epitope (amino acids 1-15) of the mycobacterial hsp65. This portion of the molecule, however, is not conserved between bacteria and eukaryotes, arguing against a role of cross-reactivity between self and mycobacterial hsps in the pathogenesis of the disease. In addition, a recent study by the same group showed that synovial T cell clones isolated from patients

with juvenile chronic arthritis turned out to recognize *E. coli* hsp60 rather than the mycobacterial hsp65 due to contamination of the recombinant mycobacterial hsp65 protein with the *E. coli* hsp60 (Life *et al*, 1993). This is in contrast to the previous finding by the same group, thus casting some doubt on the role of mycobacterial hsp65 in the pathogenesis of rheumatoid arthritis. Nevertheless, one cannot rule out the possibility that other cross-reactive host antigens could be involved, such as cartilage components which were implied in the adjuvant-induced arthritis model described above (van Eden *et al*, 1985).

1.2.4. Tuberculosis and Crohn's disease

Tuberculosis (TB) is a chronic infectious disease known to be caused by *Mycobacterium tuberculosis*. The disease is characterized by fever and weight loss, and the lesions are characterized by caseous necrosis in tuberculosis patients. The mechanism of immunity to mycobacteria is unclear. It is generally believed that immunity to mycobacteria depends on the killing of the organisms by macrophages activated by cytokines released by T helper cells, or by cytotoxic T cells through the lysis of infected macrophages containing mycobacteria (Kaufmann, 1990).

Crohn's disease (CD) is a chronic inflammatory bowel disease. The hallmark of the disease is granuloma formation, which is seen in about 70% of the patients. The aetiology of the disease is unknown. A mycobacterial aetiology has long been suspected. An extremely slow-growing mycobacterium, *Mycobacterium paratuberculosis*, has been

isolated from the gut specimens of some patients with Crohn's disease (Burnham *et al*, 1978). *Mycobacterium paratuberculosis* is a causative agent of Johne's disease in cattle, goats and deer. Johne's disease is histologically similar to Crohn's disease. However, the causative role of this mycobacterium in humans remains controversial.

1.2.5. The significance of raised level of agalactosyl IgG

The mechanisms causing the increase in agalactosyl IgG, and the significance of such an increase in the pathogenesis of these diseases, including RA, TB and CD, remain unclear. It has been suggested that the increase in agalactosyl IgG may be due to a reduction in the activity of β -galactosyltransferase in B cells, resulting in reduced transfer of galactose onto the terminal GlcNAc on IgG. Reduced β -galactosyltransferase activity has been found in circulating B cells from patients with RA (Axford *et al*, 1987), as well as in myeloma cells (Nishiura *et al*, 1990). However, it is not known what factors are involved in the regulation of this enzyme. Attempts had been made to search for a genetic control (Tomana *et al*, 1988; Delves *et al*, 1990). A single gene copy was found to encode the β 1,4-galactosyltransferase (Hollis *et al*, 1989). In a study of galactosyltransferase activator gene, no mutation on this gene was demonstrated in RA patients with reduced galactosyltransferase activity (Delves *et al*, 1990). Suggestions were also made to relate the decreased galactosyltransferase activity to the down-regulation by cytokines released during a type of immune response which was shared by the diseases with raised level of agalactosyl IgG

(Rademacher *et al*, 1988). Among these cytokines, IL-6 was chosen to be a strong candidate (Rook *et al*, 1991a), since it is a major cytokine in controlling the production of immunoglobulins from B cells (Kishimoto *et al*, 1989). Other cytokines, such as TNF, IL-1, may exert their effects via release of IL-6 (Shalaby *et al*, 1989). IL-6 has been found capable of causing changes in the activity of glycosyltransferases, including galactosyltransferase, in B cells (Nakao *et al*, 1990). Moreover, extremely high levels of agalactosyl IgG were detected in transgenic mice over-expressing the human IL-6 gene (Rook *et al*, 1991a).

The presence of raised agalactosyl IgG in patients with TB, an infectious disease caused by *Mycobacterium tuberculosis*, implied that the increase in agalactosyl IgG in patients with RA and CD may therefore be one more clue to a mycobacterial aetiology in these diseases (Rademacher *et al*, 1988). On the other hand, the increase in agalactosyl IgG was not always an indicator of infection with mycobacteria. Raised levels of agalactosyl IgG were also detected in mice with pristane-induced arthritis, in which no mycobacteria were injected, but an increased levels of anti-hsp 65 antibodies were found (Thompson *et al*, 1990; Rook *et al*, 1991a). The increase in levels of anti-hsp65 antibodies was also found to correlate strongly with the raised levels of agalactosyl IgG in mice injected with a large intravenous dose of BCG (Rook *et al*, 1990c). In patients with RA, raised levels of anti-hsp65 antibodies were also demonstrated (Bahr *et al*, 1988; Tsoulfa *et al*, 1989). It is not known whether these two phenomena, the simultaneous increases in the levels of anti-hsp65 antibodies and agalactosyl IgG, are directly related to each other or

related to the disease process independently.

It is not known whether the increase in agalactosyl IgG in disease states is only an accompanying phenomenon or involved in the pathogenesis of these diseases. The association between the levels of agalactosyl IgG and the disease activity in RA may imply the involvement of agalactosyl IgG in the pathogenesis of RA.

It has been suggested that the increase in agalactosyl IgG may facilitate immune complex formation by allowing rheumatoid factors to be bound more effectively (Parekh *et al*, 1985). It has been noted that the monoclonal anti-GlcNAc antibody (of IgM isotype) could not bind to agalactosyl IgG unless it was denatured (Rook *et al*, 1988). It was also shown that the ability of IgG molecules to bind to IgM rheumatoid factors was not changed by removing the galactose residues (Tsuchiya *et al*, 1989). In contrast, a recent study found that the binding affinity of IgM rheumatoid factors was associated with the galactosylation of IgG (Soltys *et al*, 1994).

It was also suggested that agalactosyl IgG probably tends to aggregate in a concentration-dependent manner (Parekh *et al*, 1985; Rademacher *et al*, 1988). In established RA, levels of agalactosyl IgG were found to be high in synovial fluid. The aggregation may be partly due to the presence on the Fc of a lectin-like site normally occupied by the galactosylated α 1-6 arm of the oligosaccharide at asparagine 297 (Fig. 1.1). If the α 1-6 arm is agalactosyl, this site is 'vacant' and may bind to sugars on other molecules, including other IgG molecules with glycosylated Fab moieties. Such aggregates may be biologically active, and evoke cytokine release from macrophages.

The carbohydrate-dependent effector functions of IgG may be

affected by the loss of terminal galactose residues from the oligosaccharides in the C γ 2 domain. It has been shown that depletion of galactose from human IgG resulted in the reduced ability of IgG to bind to Fc receptors on a human macrophage cell line, and to a complement component, C1q, retaining the ability to interact with IgM rheumatoid factors and to Protein A (Tsuchiya *et al*, 1989). It is not known whether other effector functions of IgG involving Fc receptor binding are also affected, such as ADCC, phagocytosis, and negatively feedback control on antibody synthesis.

1.3. Aim of the study

1. To develop a more efficient version of G(0) immunoassay to facilitate the measurement of agalactosyl IgG in a large number of serum samples from various relevant diseases.
2. To measure the levels of agalactosyl IgG in sarcoidosis with larger numbers of serum samples, and in other diseases such as Takayasu's arteritis.
3. To investigate the role of IL-6 in regulating the production of agalactosyl IgG.
4. To investigate the ability of agalactosyl IgG to bind to different types of Fc receptors *in vitro*, and to induce tolerance to a hapten *in vivo*.

Chapter 2

The Development of G(0) Immunoassay

2.1. Introduction

There is a conserved *N*-glycosylation site at asparagine 297 on the C γ 2 domain of the IgG molecule. The *N*-linked oligosaccharides are of bi-antennary complex-type. Structural analysis indicated that serum IgG from patients with rheumatoid arthritis often lacks the terminal galactose from these *N*-linked oligosaccharides, thus terminating in GlcNAc (Parekh *et al*, 1985). In other words, patients with rheumatoid arthritis have raised serum levels of agalactosyl IgG. This change in *N*-glycosylation of IgG was originally measured using a biochemical method (Parekh *et al*, 1985). The procedure involved the use of hydrazine to release IgG-associated oligosaccharides, radioactive labelling of each oligosaccharide chain, separation of an aliquot of the labelled oligosaccharide mixtures by high-voltage paper electrophoresis, and digestion of other aliquots of the labelled oligosaccharide mixtures with exoglycosidases of defined specificity, fractionation of each specifically digested oligosaccharide mixture either by gel filtration chromatography, or by a lectin-agarose affinity chromatography. Therefore, it is a very labour intensive and time consuming method, which also requires a big volume of each serum sample to purify enough IgG (about 5 mg). The measurement of percentage agalactosyl IgG [%G(0)] was simplified by the

development of an immunoassay using a monoclonal antibody directed against the terminal GlcNAc residues exposed on agalactosyl IgG molecules (Rook *et al*, 1988, Filley *et al*, 1989). The first version of the immunoassay to measure the serum level of agalactosyl IgG was called a 'dip stick' assay (Filley *et al*, 1989). The principle of the assay is to use a solid phase, i.e., nitrocellulose soaked with Protein A to capture serum IgG, of which the terminal GlcNAc of agalactosyl IgG is then detected by the biotinylated anti-GlcNAc monoclonal antibody. The nitrocellulose was cut into the shape of a comb such that the comb teeth absorbed with Protein A could be dipped into the wells of an ELISA plate to capture serum IgG. Compared with the biochemical method, the 'dip stick' assay was much simpler and more economical. However, the technique still contains tedious and time-consuming procedures and is far from ideal. For instance, it takes a long time to prepare the nitrocellulose comb and care has to be taken throughout the assay so as not to tear the nitrocellulose membrane. At the end of the assay, the optical density for each sample has to be measured individually, which makes the assay very tedious. Furthermore, the colour developed on each tooth of the nitrocellulose comb is not always even. Therefore, the technique of the immunoassay needed further improvement. A more efficient version of the immunoassay was necessary for screening a large number of serum samples in various relevant diseases.

2.2. Materials and Methods

2.2.1. Affinity purification and biotinylation of anti-GlcNAc monoclonal antibody (GN7)

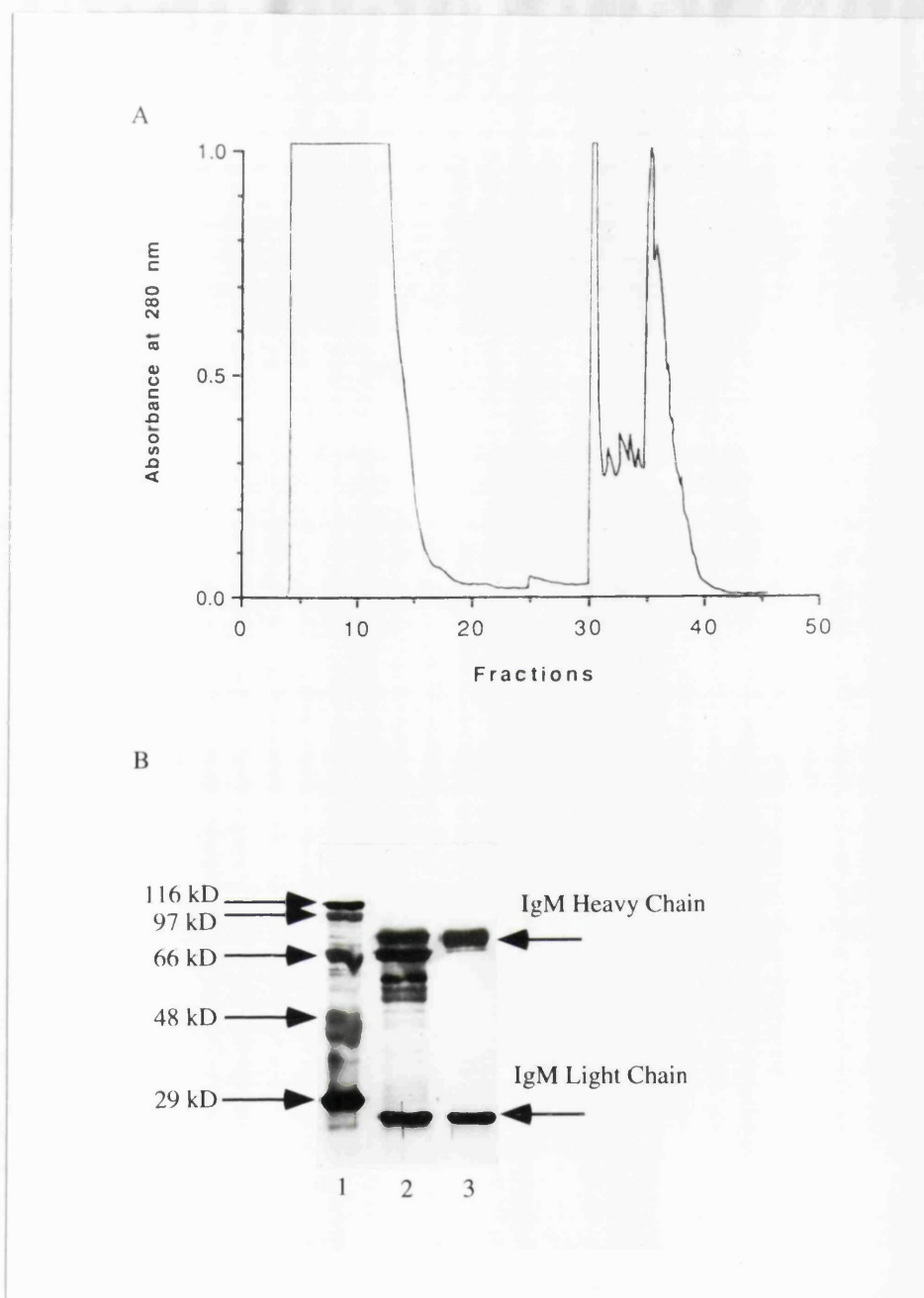
2.2.1.1. Affinity purification of anti-GlcNAc monoclonal antibody from ascites

About 5 ml uniform suspension of the GlcNAc-agarose (Sigma) was packed into a 10 ml syringe without the plunger. The packed column was supported on a stand with the tip end down and then connected to a chart recorder and a fraction collector (LKB) through tubing.

The column was equilibrated with 25 ml of PBS (pH 7.4, APPENDICES). The ascites was centrifuged, diluted with an equal volume of PBS and then filtered through 0.2 μ m filter. About 10 ml of the diluted ascites was run through the column with a flow rate of 30 ml/hr approximately. When the sample had been absorbed into the gel bed, the column was washed with 25 ml of PBS to remove all unbound protein. The bound antibody was eluted with 0.5 M *N*-acetyl-*D*-glucosamine (Sigma) solution and all fractions were collected individually. The elution profile is shown in Figs. 2.1A and 2.1B. Fractions 30 - 40 were pooled together. The column was re-equilibrated with PBS if another run was required, otherwise the column was stored in PBS containing 0.05% sodium azide at 4°C.

The purified antibody was concentrated in a dialysis tubing using Aquacide I powder (CALBIOCHEM), and then dialysed against

Figure 2.1 The purification profile of anti-GlcNAc monoclonal antibody



The anti-GlcNAc monoclonal antibodies were purified from the ascites using a GlcNAc-agarose column (A). Fractions 5-20 were the unbound protein, and fractions 30-40 eluted from the column were the purified anti-GlcNAc antibody. The purity of the eluate was checked by 10% SDS-PAGE (B). Lane 1: protein marker; Lane 2: anti-GlcNAc containing ascites; Lane 3: the purified anti-GlcNAc (fractions 30-40).

PBS overnight at 4°C. The antibody concentration was estimated by using BIO-RAD Protein Assay Kit. The purity of the purified antibody was checked by 10% SDS-PAGE (Fig. 2.1B). As can be seen, the anti-GlcNAc monoclonal antibody is of IgM isotype. Finally the purified antibody was sterilized by filtering through 0.2µm filter and stored at -20°C.

2.2.1.2. Biotinylation of purified anti-GlcNAc monoclonal antibody

The concentration of the antibody was adjusted to 1 mg/ml with PBS. The biotin (Sigma) solution was made up to 1 mg/ml in DMSO (Sigma) just before use. Then the antibody solution was mixed with biotin/DMSO solution at a ratio (v/v) of 10 : 1 in a glass container. The mixture was incubated at room temperature overnight in dark. The biotinylated anti-GlcNAc antibody was stored at 4°C for future use.

2.2.2. G(0) 'dip stick' assay

Nitrocellulose sheets were cut with a sharp scalpel into 'combs' with twelve teeth with the help of a template. These combs are spaced so that each tooth could enter a microtitre well, while the backbone of the comb was supported on the side of the wells. The entire combs were then incubated for 2-3 days in Protein A (Sigma) at 250 µg/ml in PBS at 4°C. The Protein-A-coated nitrocellulose was then washed in PBS/BSA/Tween (APPENDICES) for 2 hours at room temperature.

Serum samples were diluted 1/50 in a glycine buffer (see

Appendices) consisting of 0.1M glycine and 0.16M NaCl adjusted to pH 7.0 with 1N NaOH to reduce aggregation of IgG (Hansson, 1970). Aliquots of 250 μ l were loaded in flat-bottomed microtitre wells (Nunc) in triplicate. The Protein A-coated combs of nitrocellulose were placed in these wells and incubated at room temperature for 4 hours with occasional agitation. Then the combs were removed from the well, washed twice in PBS/BSA/Tween (see Appendices), once in PBS, and fixed in 0.5% glutaraldehyde in PBS for 30 minutes at 0°C. Fixed combs were washed again in PBS at 4°C containing 0.1 M lysine, then boiled for 5 minutes in PBS in a double waterbath to denature the IgG.

The nitrocellulose was then incubated on a rocking platform at room temperature for 3 hours in biotinylated anti-GlcNAc antibody at a dilution of 1/2000 in PBS/BSA/Tween. After careful washing the nitrocellulose was incubated for 2 hours at room temperature in an avidin/peroxidase complex (Amersham) diluted 1/500 in PBS/BSA/Tween.

Subsequently, the colour of the assay was developed by adding a mixture of hydrogen peroxide and precipitating chromogen (4-chloronaphthol, Sigma) in 5mM Tris/HCl buffer at pH 7.6 for 15 minutes. After drying, the tests were read with a transmitted light photometer adapted from a simple ELISA reader described previously (Rook and Cameron, 1981).

In each assay, a set of serum samples, the %G(0) values of which had been obtained by the biochemical procedure, were used as standards.

To calculate the results, a curve-fitting program (Dataplot, by

S.M. Fraser, Strathclyde University, Glasgow, UK) was used to plot the absorbance values, yielded by the standards in the 'dip stick' assay, against the %G(0) results previously determined biochemically. A log-linear correlation was found. The %G(0) of the unknown samples were then interpolated from the log-linear curve.

2.2.3. SDS-PAGE and Western blotting

2.2.3.1. Preparation of SDS-PAGE gel

The method used for preparation of polyacrylamide gels was essentially that of Laemmli (1970). The gel apparatus used was Mighty Small minigel (Hoeffer Scientific Instrument). The glass and aluminum plates were cleaned thoroughly with detergent and rinsed with water and ethanol and then dried with tissues. The spacers used were 0.75 mm thick. The plates were clamped to the electrophoresis core which was then placed on a glass plate. The bottom of the plates was sealed with 1% molten agarose. The separating gel solution was prepared as follows and loaded between the plates using plastic Pasteur pipettes.

After loading the separating gel, a few drops of water-saturated butanol was added on top of the gel solution to remove air bubbles. Then the gel was allowed to polymerize at room temperature for about 20 minutes. When the separating gel was set, butanol was poured off and the gel was washed with dH₂O using a wash bottle. The stacking gel was prepared by mixing the following solutions and loaded on top of the separating gel with a plastic pasteur pipette and the combs were

inserted immediately.

When the stacking gel had set, the combs were removed carefully and the sample wells were rinsed with dH₂O to remove unpolymerized gel. Electrophoresis buffer was added to the electrophoresis core and chamber.

Separating gel recipe

	12% gel (1 gel)	6% gel (1 gel)
30% acrylamide/ bis-acrylamide (19:1)	2 ml	1 ml
dH ₂ O	1.7 ml	2.7 ml
1M TrisHCl (pH 8.8)	1.2 ml	1.2 ml
10% SDS	50 µl	50 µl
TEMED	7.5 µl	7.5 µl
10% APS	75 µl	75 µl

Stacking gel recipe

30% acrylamide/ bis-acrylamide (19:1)	0.4 ml
dH ₂ O	1.4 ml
0.5M TrisHCl (pH 6.8)	0.6 ml
10% SDS	25 µl
TEMED	3.5 µl
10% APS	35 µl

Samples were prepared by mixing with an equal volume of 2x

sample buffer and boiled for 3 minutes. The samples were loaded together with a set of molecular weight markers (Sigma), using the super-fine tips. The gels were run at 15 mA per gel using a power pack (LKB). When the samples had run to the bottom, the power pack was switched off and the electrophoresis buffer was poured off. The gel apparatus was disassembled and the gels were either stained by Coomassie blue or electrotransferred to nitrocellulose.

For Coomassie stain, the gel was incubated in Coomassie staining solution (see APPENDICES) for 30 minutes and then destained with destaining solution (see APPENDICES) for 1 hour with several changes of destaining solution.

2.2.3.2. Western blotting

To transfer the proteins from gels to nitrocellulose membrane, the nitrocellulose sheet (Amersham) and 3 MM Whatman paper were cut to the size slightly larger than the gel. The gel was loosened from the sides using a spacer. The nitrocellulose and 3 MM Whatman paper were wetted with blot buffer (see APPENDICES) and the gel was transferred onto the nitrocellulose sheet, another piece of 3 MM Whatman paper wetted with blot buffer was placed on top of the gel. Two pieces of sponge soaked with blot buffer were placed both on top and bottom of the gel nitrocellulose sandwich. The above assembly was transferred to the blotting chamber containing blot buffer, with the nitrocellulose side towards the anode. Transfer was done at 50 V for 1 hour according to Towbin *et al* (1979).

After the transfer, the nitrocellulose membrane was stained with

0.2% Ponceau S (Sigma) in 3% trichloroacetic acid (BDH) to visualize the protein bands on the nitrocellulose. A photocopy was then made to keep as a reference. The Ponceau S dye was washed off with dH₂O. Nonspecific binding was blocked with PBS/BSA/Tween for 2 hours at room temperature. Biotinylated anti-GlcNAc monoclonal antibody was added to the membrane at 8 µg/ml in PBS/BSA/Tween. After 2 hours incubation with gentle agitation at room temperature, the membrane was washed 3 times for 10 minutes in PBS/Tween. Streptavidin-horseradish peroxidase (DAKO) at 1:500 dilution in PBS/BSA/Tween was added and incubated for a further 2 hours at room temperature. Following 5 washes with PBS/Tween, the enzyme substrate solution (0.5 ml of 0.2M Tris-HCl pH7.6, 4.0 ml of 3 mg/ml 4-chloro-1-naphthol in methanol, 19.5 ml distilled water, 10 µl of 30% H₂O₂) was added to the nitrocellulose membrane and incubated for 10 minutes at room temperature. The colour reaction was terminated by rinsing the blot with distilled water.

2.2.4. Details of samples

The serum samples whose %G(0) values had been biochemically determined were kindly provided by Biochemistry Unit at Oxford University.

The serum samples from patients with rheumatoid arthritis and the control samples of patients with no rheumatoid arthritis but with other soft tissue disorders were provided by Dr van Zeben at Leiden University Hospital in the Netherlands. The diagnosis of RA was made according to the American Rheumatism Association (ARA)

criteria for definite RA.

2.3. Results

2.3.1. G(0) plate assay

To improve the 'dip stick' assay, the following modifications were made. Firstly, a flat-bottomed microtitre ELISA plate (Nunc immunoplate Maxisorb) was used as a solid phase to absorb Protein A instead of nitrocellulose membrane. Secondly, the denaturation of IgG was carried out initially by heating the plate in a microwave and then by floating the microtitre plate in a pre-heated waterbath at 85°C. Thirdly, the optical densities generated at the end of the assay were read by an ELISA reader. Fourthly, the parameters involved were standardized as follows.

2.3.1.1. Standardization of the protocol

The concentration of Protein A

To determine the appropriate concentration of Protein A in the G(0) plate assay, a microtitre plate was coated with 50 µl per well of Protein A (Sigma) at various concentrations, ranging from 0.312 - 10 µg/ml, in PBS (pH 7.4). The plate was incubated overnight at 4°C, and then blocked with 100 µl per well of PBS/BSA/Tween at 37°C for 1 hour. Three serum samples with different %G(0) were diluted 1: 50 in the glycine buffer (0.1M glycine and 0.16M NaCl, pH 7.0). After washing the plate with PBS/Tween, 50 µl of the diluted sera was

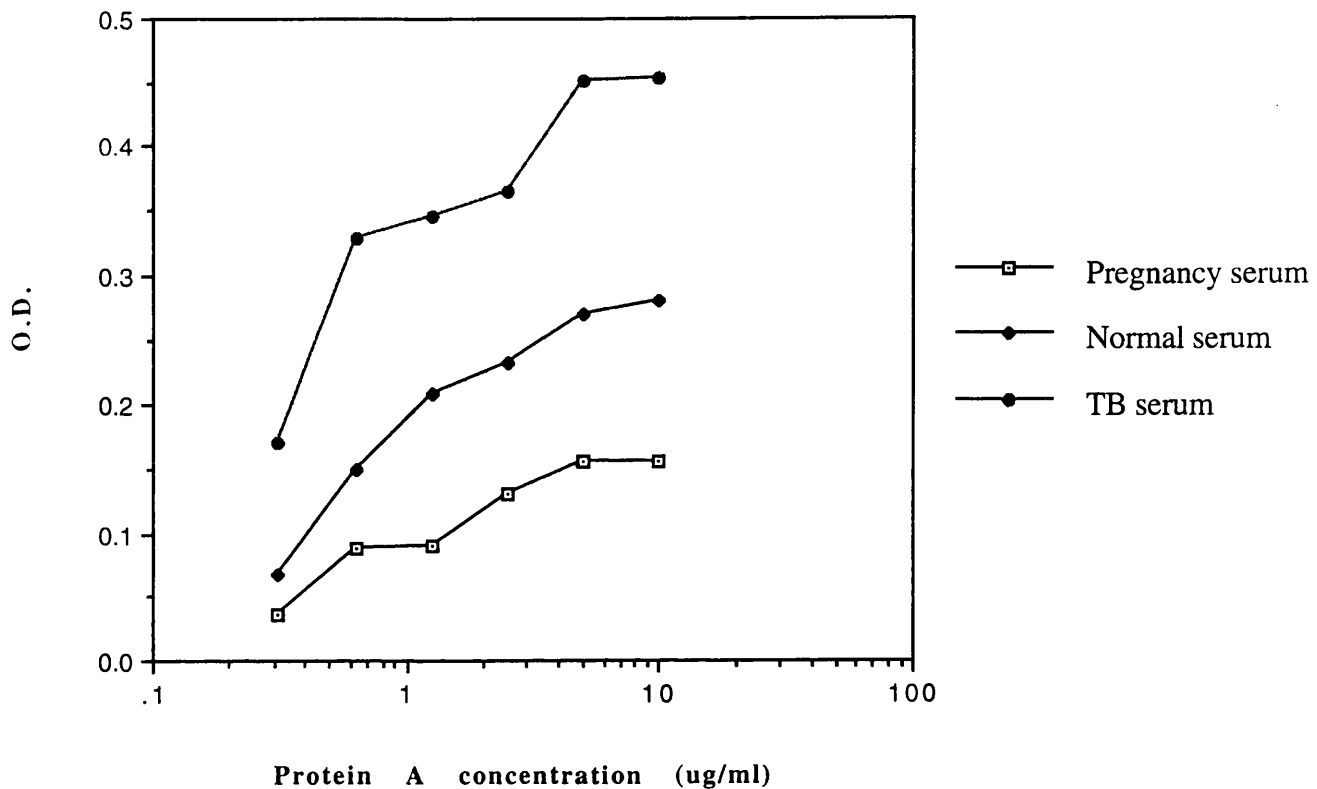
added to each well in duplicate, and incubated at 37°C for 2 hours. The plate was then washed twice with PBS/Tween and once with PBS only, and heated in a microwave at full power for about 1 - 2 minutes. After cooling down, 50 µl per well of the biotinylated anti-GlcNAc antibody at 2 µg/ml in PBS/BSA/Tween was loaded, and the plate was incubated at 4°C overnight. Then the plate was washed three times with PBS/BSA/Tween, and added with 50 µl per well of peroxidase-conjugated streptavidin (Dako) diluted 1:1000 in PBS/BSA/Tween, and incubated at 37°C for 1 hour. The colour was developed using ABTS as substrate (APPENDICES), and the optical density was read with an ELISA reader.

The Protein A titration curve was shown in Fig. 2.2. As can be seen, the curves reached a plateau at about 5 µg/ml of Protein A. However, the concentration of Protein A was chosen to be 2.5 µg/ml, because no apparent difference can be seen between coating the plate with 5 µg/ml and 2.5 µg/ml of Protein A in other experiments when the serum samples were diluted 1:100 instead of 1:50 (data not included).

Serum dilution

To determine the appropriate dilution of serum samples in the G(0) plate assay, a plate was coated with Protein A at 2.5 µg/ml overnight at 4°C. Three serum samples with varying %G(0) were doubly diluted from 1:50 downwards in the glycine buffer (pH 7.0). Then 50 µl of each dilution was loaded in duplicate into each well of the plate coated with Protein A. The plate was incubated at 37°C for 2 hours. After twice washed with PBS/Tween and once with PBS, the

Figure 2.2 Titration of Protein A in G(0) plate assay



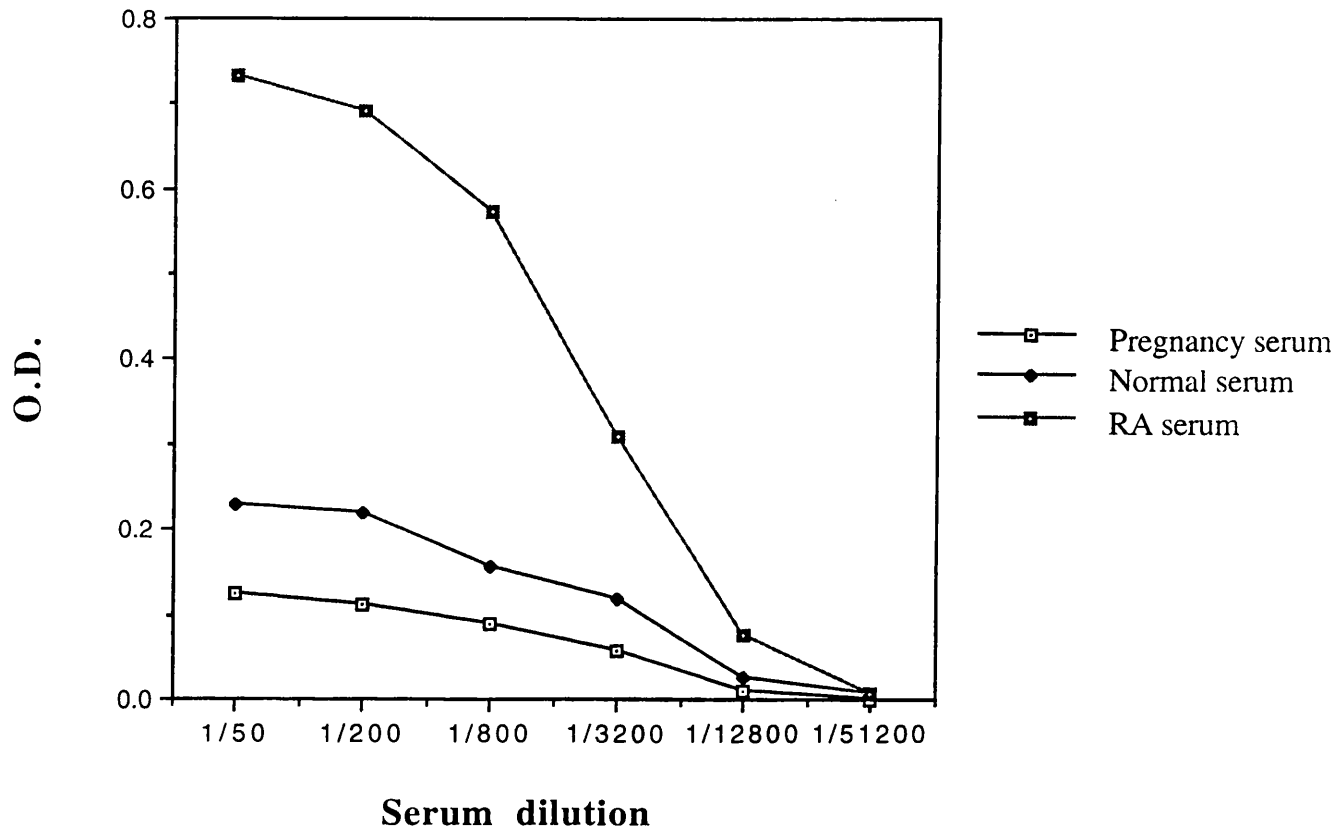
Sera of low (pregnancy), normal, and high (tuberculosis) %G(0) were tested in the G(0) plate assay. All variables were held constant as described in the text, except for the concentration of Protein A used to coat the wells, which was varied between 0.31 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$.

plate was heated by floating in a waterbath at 85°C for about 7-8 minutes. The rest of the assay was carried out the same as described above. The concentration of the anti-GlcNAc was used at 2 µg/ml and streptavidin was used at 1:1000. The optical densities obtained were plotted against the serum dilutions, as illustrated in Fig. 2.3. It was shown that the optical densities began to decline dramatically at 1:200 serum dilution. To assure the saturation of Protein A in the wells of a plate, serum dilution at 1:100 was subsequently used in the G(0) plate assay.

Concentration of anti-GlcNAc antibody

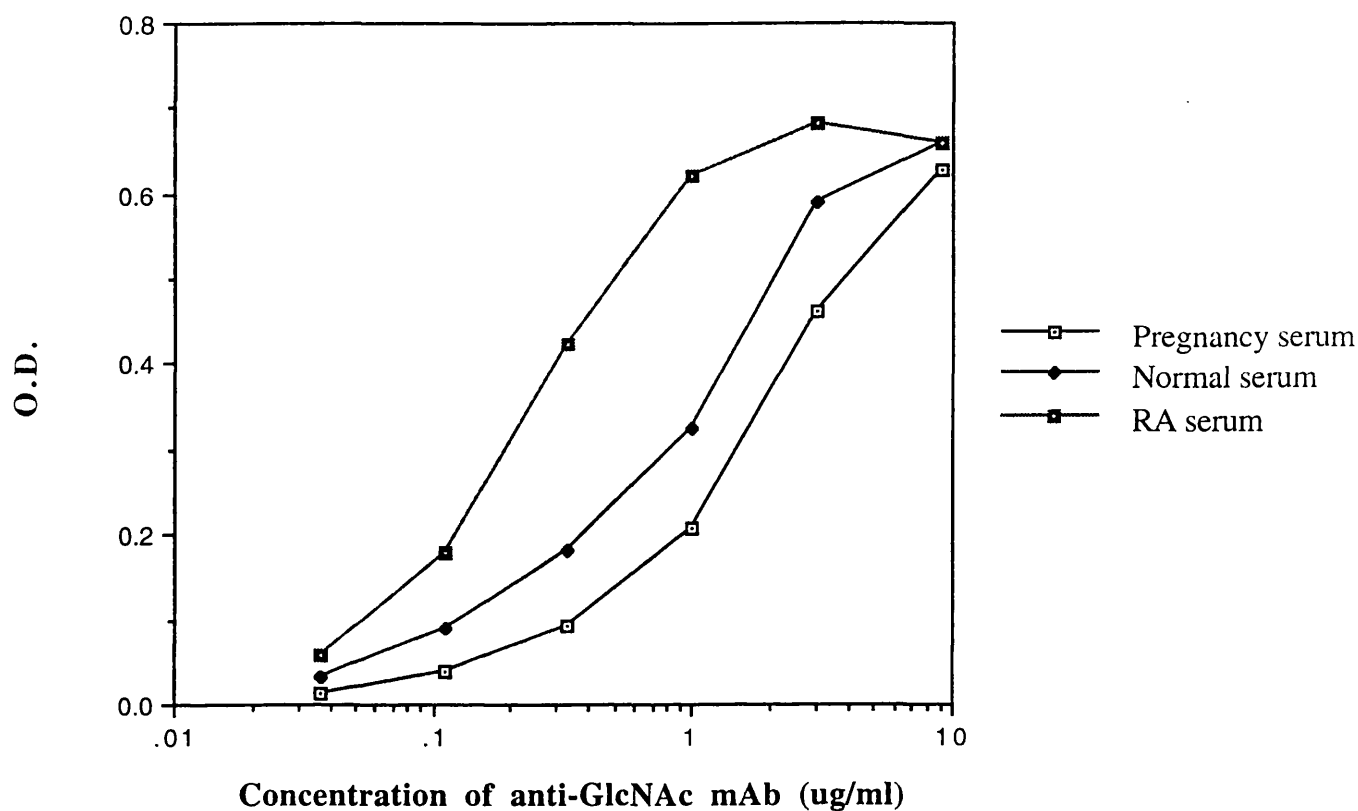
To find out the optimal concentration of anti-GlcNAc in the G(0) plate assay, a plate was coated with 2.5 µg/ml of protein A, and then loaded with 1:100 diluted serum samples. The experimental procedure was essentially the same as described above. After heating the plate in a waterbath at 85°C for 7-8 minutes, the plate was incubated with 50 µl per well of the biotinylated anti-GlcNAc antibody at various concentrations, ranging from 0.05 µg/ml to 10 µg/ml. The anti-GlcNAc antibody titration curves of three samples were shown in Fig. 2.4. It was found that the anti-GlcNAc titration curve of a RA serum [known to have high %G(0)] reached a plateau at about 1 µg/ml of anti-GlcNAc antibody, while the titration curves of the other two samples [known to have lower %G(0)] were still steadily increasing. The concentration of anti-GlcNAc antibody was chosen at 2 µg/ml in the G(0) plate assay. This would ensure the optimal discrimination between samples in %G(0).

Figure 2.3 Dilution of serum samples in G(0) plate assay



Sera of low (pregnancy), normal, and high (rheumatoid arthritis) %G(0) were tested in the G(0) plate assay. All variables were held constant as described in the text, except for the dilution of the serum, which was varied between 1/50 and 1/51200.

Figure 2.4 Titration of the anti-GlcNAc antibody in G(0) plate assay



Sera of low (pregnancy), normal, and high (rheumatoid arthritis) %G(0) were tested in the G(0) plate assay. All variables were held constant as described in the text, except for the concentration of anti-GlcNAc, which was between 0.31 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$.

Heating time

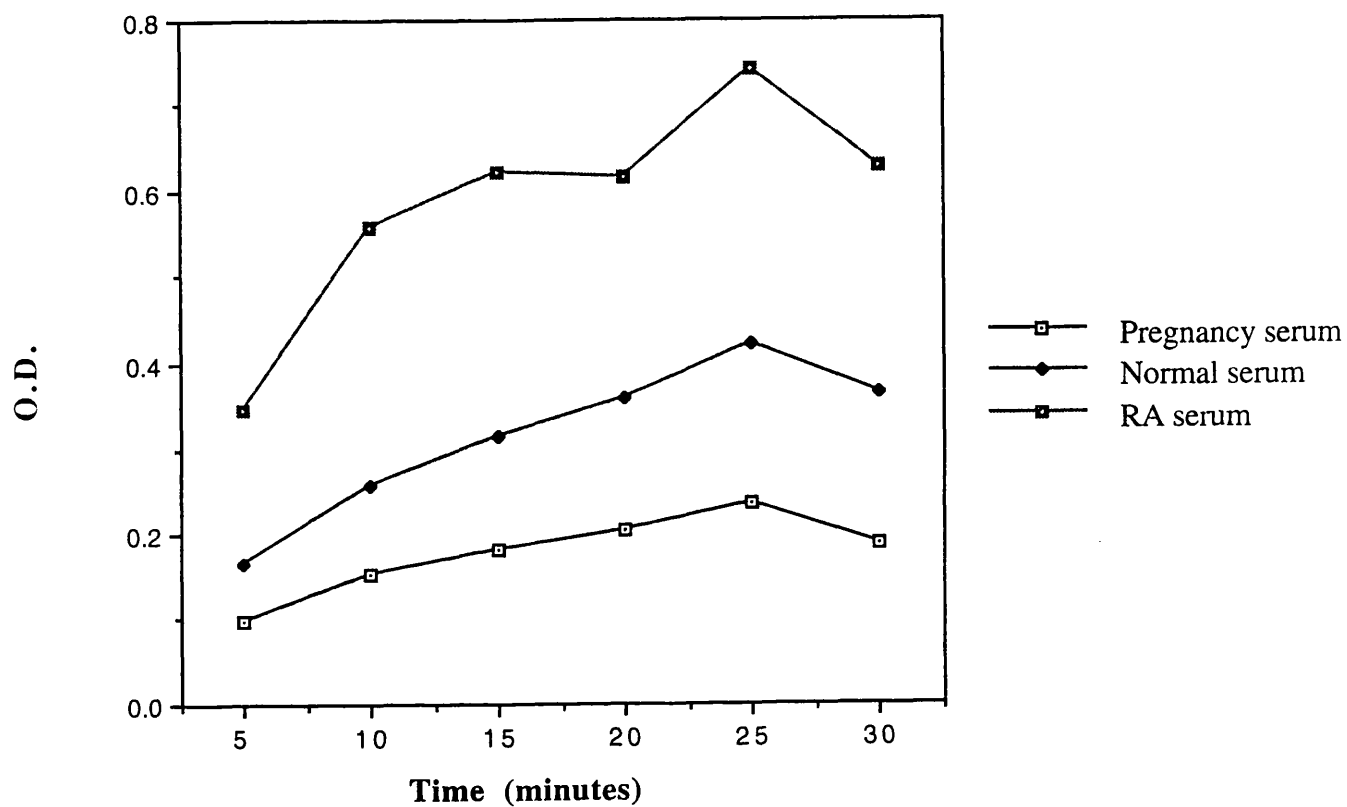
To find out the appropriate heating time for IgG denaturation in G(0) plate assay, several plates with Protein-A-bound-IgG were floated in a waterbath at 85°C for various lengths of time between 5 - 30 minutes. In this assay, the plates were coated with Protein A at 2.5 µg/ml, and loaded with sera diluted at 1:100, with the biotinylated anti-GlcNAc at 2 µg/ml. Other procedures in the assay were the same as described above. The optical densities obtained were plotted against the time of heating. As shown in Fig. 2.5, heating for about 10 minutes in a waterbath at 85°C was long enough to generate a strong signal. To avoid overheating the plastic ELISA plate, which caused distortion and cloudiness, and also ensure the production of a strong signal, heating the plate for 8 minutes in a waterbath at 85°C was used to denature IgG in future G(0) plate assay.

2.3.1.2. The protocol of G(0) plate assay

A 96-well flat-bottomed ELISA plate (Nunc immunoplate Maxisorb) was used in the assay. Except the edge wells, each well of the plate was coated with 50 µl of protein A (Sigma) solution at 2.5 µg/ml in PBS (pH 7.4). The plate was kept in a damp box and incubated at 4°C overnight.

The next day, the plate was emptied and any remaining protein-binding sites were blocked by incubating 100 µl per well of PBS/BSA/Tween at 37°C for 1 hour. Then the plate was washed three times by filling up the wells with PBS/Tween using a multi-channel pipette followed by incubation at room temperature for 5-10 minutes

Figure 2.5 Time course of heat denaturation of IgG in G(0) plate assay



Sera of low (pregnancy), normal, and high (rheumatoid arthritis) %G(0) were tested in the G(0) plate assay. All variables were held constant as described in the text, except for the duration of heating at 85°C which was varied between 5 minutes and 20 minutes.

and bang-drying on a pack of tissue towels. The human serum samples were diluted at 1 : 100 in a glycine buffer consisting of 0.1M glycine and 0.16M NaCl, adjusted to pH 7.0 with 1N NaOH. Then 50 μ l of the diluted sera was added to each well in duplicate. The plate was incubated at 37°C for 2 hrs in a damp box. After incubation, the plate was washed twice with PBS/Tween and twice with PBS only as above. Then 50 μ l of PBS, which was pre-warmed to room temperature, was added to each well (include the edge wells) and the plate was floated in a pre-heated waterbath at 85°C for 8 minutes to denature the IgG. Care was taken to make sure that there was no air bubbles trapped underneath the plate. After cooling the plate to room temperature and bang drying, 50 μ l of the biotinylated GN7 antibody solution at 2 μ g/ml diluted in PBS/BSA/Tween was loaded to each well. The plate was then kept at 4°C in a damp box overnight.

On the third day, the plate was washed three times with PBS/Tween as above and then incubated with 50 μ l per well of peroxidase-conjugated streptavidin (Dakopatts) diluted to 1 : 1000 in PBS/BSA/Tween, at 37°C for 1 hour. Finally, after further three washes with PBS/Tween, each well of the plate was added with 50 μ l of ABTS substrate solution (APPENDICES) to develop the colour. After 15-20 minutes incubation at 37°C in dark, the colour reaction was stopped by adding 50 μ l per well of sodium fluoride (Sigma) solution (APPENDICES). The optical density values were determined by an ELISA reader at 630 nm with a reference wavelength of 490 nm.

2.3.2. Correlation between the anti-GlcNAc binding in G(0) plate assay and the %G(0) obtained biochemically

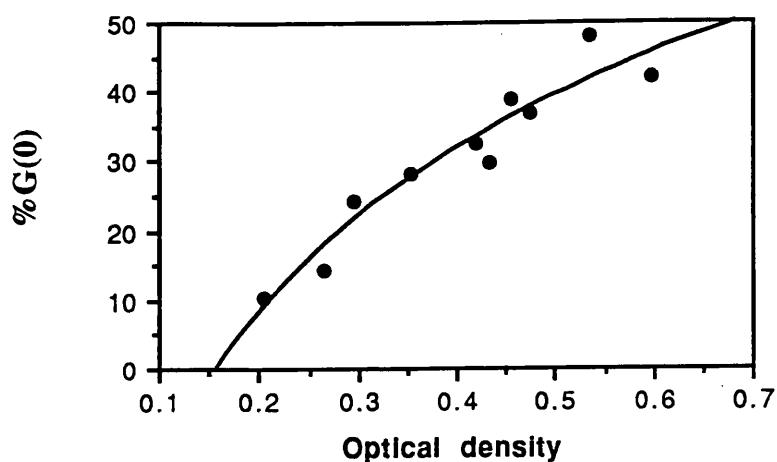
When a set of standards with known %G(0) determined by biochemical method were tested by 'G(0) plate assay', as shown in Fig. 2.6, the binding of anti-GlcNAc (absorbances) is positively correlated with the percentage of oligosaccharide chains terminated exclusively in GlcNAc (%G0). A log-linear relationship ($r=0.92$) was found between the absorbance values and the %G(0) values using the same curve-fitting program (Dataplot) as adopted in the 'dip stick' assay. This log-linear correlation was subsequently used as a calibration curve to interpolate the %G(0) of the unknown samples also using the Dataplot program.

When a large number of unknown samples were tested using the G(0) plate assay, a serum free control and 4 human standards sera with known %G(0) were included in each assay. The four standards sera were from a pregnant women, a normal donor, a tuberculosis and a rheumatoid arthritis patients. The %G(0) values of the 4 standards are between 10% - 42% which obtained biochemically. The log-linear standard curve (Fig. 2.7) generated from every plate, plotted between the absorbance values and the %G(0) values of the standards, was then used to interpolate the %G(0) values of the unknown samples tested in the same plate.

2.3.3. Validation of the G(0) plate assay for %G(0) measurement

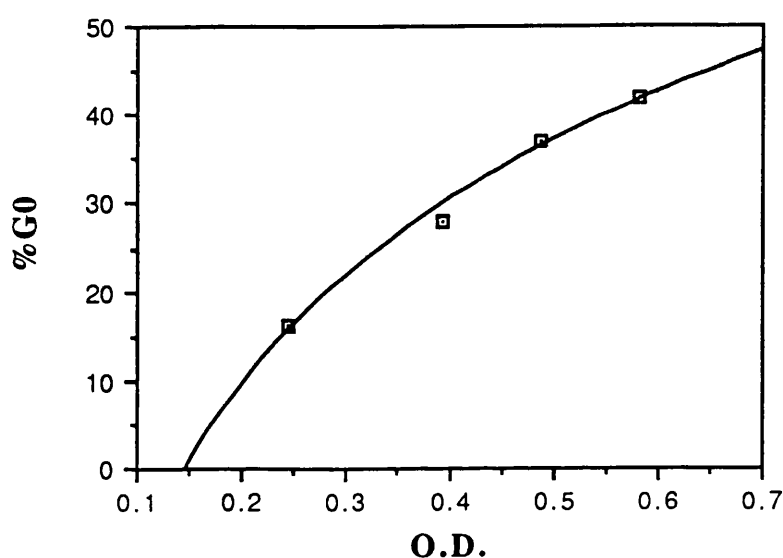
To find out how accurate the G(0) plate assay is in the

Figure 2.6 Correlation between the anti-GlcNAc binding and the %G(0) obtained using biochemical method



Ten serum samples were tested independently for agalactosyl IgG using the biochemical method and the G(0) plate assay. The percentage of agalactosyl IgG obtained using biochemical method was plotted against the anti-GlcNAc binding (O.D.) in G(0) plate assay. A log-linear relationship is shown.

Figure 2.7 Standard curve used in G(0) plate assay



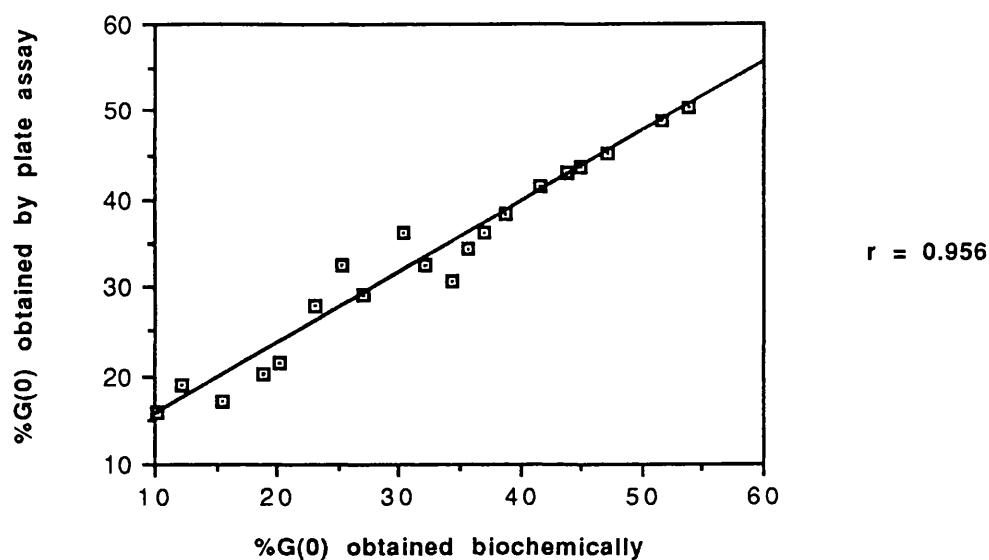
In the established G(0) plate assay, four human sera with known %G(0) were included in each run of the assay as standards. They are a pregnancy serum [10.2% G(0)], a normal serum [28.1% G(0)], a tuberculosis serum [36.9% G(0)], and a rheumatoid arthritis serum [42% G(0)]. The %G(0) were plotted against the optical densities of the four standards obtained in G(0) plate assay. The %G(0) of the unknown samples were interpolated from this standard curve.

measurement of %G(0) in serum samples, in comparison with the biochemical method, a new set of 20 serum samples were tested blind both by G(0) plate assay in this lab and by biochemical method in Oxford. In the G(0) plate assay, the %G(0) values of the 20 samples were read off the log-linear standard curve (Fig. 2.7) generated from each plate assayed. When the %G(0) values produced by G(0) plate assay were plotted against the %G(0) values obtained biochemically, as shown in Fig. 2.8, the two sets of the results were closely correlated, and the correlation coefficient was 0.956.

2.3.4. Estimation of %G(0) in patients with RA by the G(0) plate assay

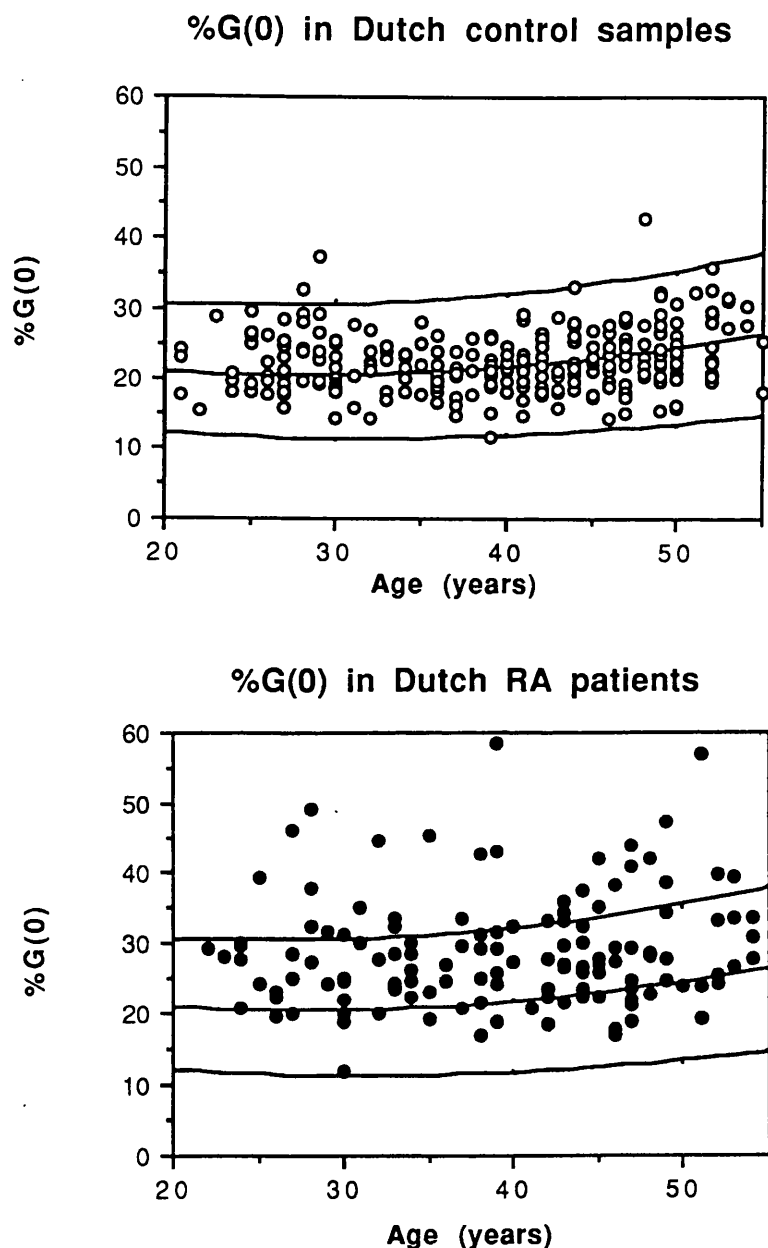
The percentages of G(0) in all serum samples, which include 127 female patients with definite RA and 265 Dutch controls with other soft tissue rheumatic disorders or osteoarthritis, were measured blind using the G(0) plate assay. In consideration of the age variation, the %G(0) results were demonstrated as shown in Fig. 2.9. It was found that the %G(0) in most of controls (except 3 individuals) fell within normal range; whereas the %G(0) in patients with RA were all elevated compared to the controls, and about 34 patients had an abnormally high level of agalactosyl IgG. The clinical significance of this study was analysed in detail by the Dutch group and the results have recently been published (van Zeben *et al*, 1994).

Figure 2.8 Validation of the G(0) plate assay for %G(0) measurement



A new set of 20 serum samples were tested blind using the established G(0) plate assay. The %G(0) were read off the log-linear standard curve, which were then plotted against the %G(0) values of these samples obtained using the biochemical method. The two sets of the results were extremely close, with a correlation coefficient equal to 0.956.

Figure 2.9 Estimation of %G(0) in patients with RA by G(0) plate assay

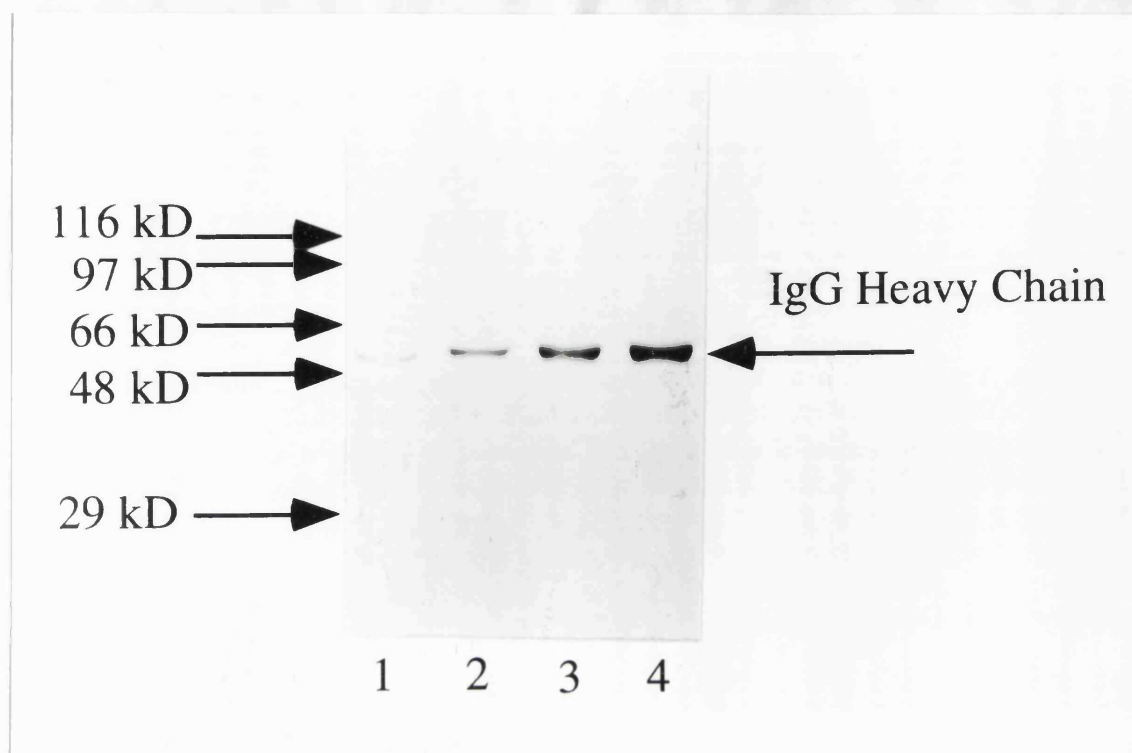


The percentages agalactosyl IgG of 265 control samples and 127 patients with rheumatoid arthritis were plotted against the age of the donor. The middle line indicates the mean percentage of agalactosyl IgG for normal donors of the same age. The area in between of the top and the bottom lines shows two standard deviations either side of the mean. About 34 patients with rheumatoid arthritis fall outside the 2x SD.

2.3.5. Detection of G(0) using Western blotting

The four human serum samples, used as standards in the G(0) plate assay, were also tested using biotinylated anti-GlcNAc monoclonal antibody by Western blotting technique. In the SDS-PAGE gel, the four serum samples (mixed with 2x sample buffer and boiled) were loaded in different volume to obtain the same quantity of serum IgG (2 µg) in each lane. After blotting, the GlcNAc residues on agalactosyl IgG molecules of all samples were identified by the biotinylated anti-GlcNAc monoclonal antibody. The blot (Fig. 2.10) clearly demonstrated the variations of %G(0) among these four samples, i.e. pregnancy IgG being the lowest (10.2%) and RA IgG being the highest (42%), which is in keeping with the results obtained by biochemical and plate assay. Meanwhile, it was noteworthy that the anti-GlcNAc antibody almost exclusively bound to GlcNAc on the heavy chains of IgG. However, because Western blot method is no simpler than the newly developed plate assay, the subsequent determination of agalactosyl IgG in patients samples was done with the plate assay.

Figure 2.10 Detection of agalactosyl IgG using Western blotting



The four standards used in G(0) plate assay were tested using biotinylated anti-GlcNAc antibody in Western blotting. Lane 1: pregnancy IgG [10.2% G(0)], Lane 2: normal IgG [28.1% G(0)], Lane 3: tuberculosis IgG [36.9% G(0)], Lane 4: rheumatoid arthritis IgG [42% G(0)]. In each lane 2 μ g of IgG was loaded. The blot was incubated with 8 μ g/ml of biotinylated anti-GlcNAc antibody. The variation in %G(0) of the four standards was clearly demonstrated. The anti-GlcNAc antibody bound exclusively to the heavy chains of IgG.

2.4. Discussion

In this study, a new, more efficient G(0) immunoassay, called the G(0) plate assay, was developed from the 'dip stick' assay. The procedure is very much like an ordinary ELISA, except the heating step. The G(0) plate assay takes only three days, and in the first day all one needs to do is to coat the plates with Protein A. However, the 'dip stick' assay covers at least 5 days, and in the first day one needs to take a couple of hours to shape the nitrocellulose combs, which is a tedious task and the nitrocellulose is brittle and breaks easily. Moreover, at the end of the G(0) plate assay, the colour developed in the wells of the plates can be automatically read by an ELISA reader within a few minutes; whereas for the 'dip stick' assay, the colour developed on each teeth of the nitrocellulose comb is not always even and has to be read manually. The new G(0) plate assay has made it possible to determine the levels of agalactosyl IgG in large numbers of patients samples economically in a short time.

In the G(0) immunoassay, a unique step is the denaturation of IgG by heating, because the anti-GlcNAc monoclonal antibodies only bind to denatured IgG (Rook *et al*, 1988). In the pilot experiments of G(0) plate assay, denaturation was initially tried in a microwave (data not shown). However, because of the uneven heating in the microwave, denaturing in a pre-heated waterbath was then tested. It was found that the plate was evenly heated except the edge wells, in which the signals (colour developed) obtained were slightly lower than those in the rest of the wells of a plate (data not shown). Therefore the edge wells of a plate were not used in the future assay.

Meanwhile, it was also found that acceptable signals can be obtained by floating the plate in a waterbath at around 85°C for a short period, but without overheating the plate. In this study, 8 minute-heating in a waterbath at 85°C was chosen to denature IgG, which seems to be the minimal time needed to generate strong signals. This 8 minute heating appeared to be a reasonable time required, and heating slightly longer, i.e., about 10 minutes could also be acceptable (Fig. 2.5).

In the G(0) plate assay, serum IgG molecules were captured by Protein A coated on a microtitre plate. In order to obtain approximately the same number of IgG molecules per well, serum dilution at 1:100 was used to ensure the saturation of Protein A in each well. Subsequently, the terminal GlcNAc exposed on IgG molecules were detected by the anti-GlcNAc monoclonal antibody of IgM isotype. The reading is therefore likely to be influenced by the terminal GlcNAc's on G(1) oligosaccharides, as well as those on G(0) oligosaccharides. Moreover, since anti-GlcNAc antibody is an IgM and therefore multivalent antibody, its binding is likely to be influenced by whether both or only one of the oligosaccharides is G(0) on a given IgG molecule. In contrast, the biochemical procedure measures the percentage of the oligosaccharides that has no galactose on either arm. It is not influenced by the terminal GlcNAc on G(1) oligosaccharides, and cannot take into account the pairing of oligosaccharides bearing 0, 1 or 2 galactose residues on individual molecules of IgG, since the oligosaccharides are stripped from the protein before they are sequenced. Thus, the G(0) plate assay and the biochemical method do not precisely measure the same thing. This may be the reason why the absorbance values obtained in the G(0)

plate assay and the %G(0) obtained biochemically are correlated logarithmically rather than linearly.

During standardization of the concentration of anti-GlcNAc antibody (Fig. 2.4), it was found that the anti-GlcNAc titration curve of the serum with high %G(0) reached the plateau earlier ($\sim 1 \mu\text{g/ml}$) than the other curves of the sera with lower %G(0), thus leading to the loss of the discrimination between samples in %G(0). The explanation for this phenomenon may be related to the fact that the anti-GlcNAc antibody is of IgM isotype (Fig. 2.1B), a huge pentamer. It is possible that, in the assay plate, when only few of the Protein-A-bound IgG are agalactosyl (i.e. pregnancy IgG), the exposed terminal GlcNAc residues are only scattered in the well; however, when a lot of the Protein-A-bound IgG are agalactosyl (i.e. RA IgG), the exposed terminal GlcNAc residues are sitting crowded in the well. Thus there seems to be more space available between the exposed GlcNAc residues in the former case for the large IgM anti-GlcNAc antibodies to rotate than that is in the latter case. Therefore the wells of the latter case are quickly crowded with the IgM anti-GlcNAc antibodies, no more space are left to fit in more anti-GlcNAc antibody molecules, even^{if} there are more of agalactosyl IgG available. In the G(0) plate assay, the concentration of anti-GlcNAc antibody was chosen at $2 \mu\text{g/ml}$, which may be a point where the wells containing more agalactosyl IgG are getting crowded. If this is the case, the working mechanism of the G(0) plate assay would possibly be more straight forward if the anti-GlcNAc antibodies were IgG isotype, and also the G(0) plate assay could be more sensitive. Nevertheless, the results [%G(0)] produced by the established G(0) plate assay are well

correlated with those obtained by the biochemical method (Fig. 2.8). Moreover, the elevation in %G(0) in patients with RA was also demonstrated in this study using G(0) plate assay, confirming the finding by Parekh *et al* (1985). Further, the variation in %G(0) in different samples was also confirmed by Western blotting using anti-GlcNAc antibodies. The binding of the anti-GlcNAc antibodies was demonstrated exclusively on IgG heavy chains (Fig. 2.10).

On the other hand, although the G(0) plate assay is simple, rapid, and efficient in screening a large number of samples, it does not produce detailed structural information which can only be obtained by the biochemical method.

Chapter 3

Raised Agalactosyl IgG in Patients with Sarcoidosis and Takayasu's Arteritis

3.1. Introduction

3.1.1. Sarcoidosis

Sarcoidosis is a multisystem disorder characterized by the presence of multiple, non-caseating granulomata in involved tissues. The disease predominantly affects young adults of either sex, and it may affect almost any tissue in the body. Most frequently, sarcoidosis presents with bilateral hilar adenopathy, pulmonary infiltration, and skin and eye lesions. Sarcoidosis occurs worldwide, but its prevalence, clinical manifestations, and outcome vary widely in different areas (Tierstein and Lesser, 1983).

The cause of sarcoidosis is unknown. However, the histological similarity between this disorder and tuberculosis has led some investigators to the view that sarcoidosis might be an unusual form of tuberculosis. Over the years, researchers in many fields have attempted to elucidate this possible relationship with *M. tuberculosis*, as well as with other infective agents in sarcoidosis. Evidence from a variety of studies is in favour of the idea that mycobacteria are possible causative agents in sarcoidosis. An elegant animal study performed by Mitchell and Rees demonstrated a transmissible agent

from human sarcoid tissue (Mitchell and Rees, 1969). Thus 6 of 11 mice inoculated with human sarcoid lymphnode homogenates showed the histological characteristics of sarcoidosis; whereas mice inoculated with non-sarcoid lymph node homogenates did not. In further studies, these authors were able to culture mycobacteria with the characteristics of *M. tuberculosis* from the mouse-sarcoid passage tissues (Mitchell *et al*, 1976; Mitchell and Rees, 1981). In another study, a cell-wall defective form of mycobacterium (mycospheroplasts) was isolated from granulomatous skin-biopsy samples from three of eight sarcoid samples tested (Graham *et al*, 1988). The recent applications of Polymerase Chain Reaction (PCR) and DNA hybridisation techniques have resulted in the demonstration of mycobacterial DNA in tissue and lavage samples from sarcoid patients, but with different levels of positivity (Saboore *et al*, 1992; Fidler *et al*, 1993; Bocart *et al*, 1992; Thakker *et al*, 1992; Mitchell *et al*, 1992).

3.1.2. Takayasu's arteritis

Takayasu's arteritis is a chronic granulomatous pan-arteritis primarily affecting the aorta and its primary branches. Varying degrees of narrowing and occlusion develop in the involved segments of the elastic arteries, leading to a wide range of symptoms. It has been given other names, such as pulseless disease, aortic arch syndrome, non-specific aortoarteritis, reversed coarctation, Martorell's syndrome and so on. The predominant clinical features are reduction of amplitude of peripheral arterial pulses, vascular bruits, and raised

blood pressure. This disease preferentially affects females (female : male ratio 8:1) below the age of 40 years (range 15-45 years). Cases of Takayasu's arteritis have been described in countries from all parts of the world. It is relatively rare in Great Britain but not uncommon in Asia, Mexico, and the Far East (Fiessinger *et al*, 1982).

The aetiology of the disease still remains obscure after its original description more than one hundred years (Savory, 1856). However, there have been a number of reports suggesting a possible role of *Mycobacterium tuberculosis* infection in the cause of the disease. The similarity between the tuberculous lesion and the aortic lesion has been noted (Nasu, 1962; Kinare, 1969). Positive PPD reaction was shown in most patients with Takayasu's arteritis investigated (Lupi-Herrera *et al*, 1972). Moreover, patients with Takayasu's arteritis have frequently been found to have a previous tuberculosis infection (Kinare, 1969; Lupi-Herrera *et al*, 1977). Yet so far no microorganisms have been demonstrated in the lesions.

In addition, attempts were also made to relate Takayasu's arteritis to an autoimmune disorder, such as rheumatoid arthritis (Ask-upmark 1954; Falicov and Cooney, 1954; Sanching and Welin 1961), systemic lupus erythematosus, polymyositis (Miller *et al*, 1962), and scleroderma (Roth and Kissane, 1964). These assumptions are based on the demonstration of circulating antiaorta antibodies (Ito, 1966), immune complexes (Giotoku *et al*, 1984), antinuclear antibodies and rheumatoid factors (Hall *et al*, 1985) in some patients with Takayasu's arteritis. However, there is no evidence as yet to show that these autoantibodies are directly pathogenic. It is possible that these autoimmune phenomena are secondary to mycobacterial infections

(Schoenfeld and Isenberg, 1988).

The discovery of raised agalactosyl IgG only in a restricted group of diseases, including tuberculosis, rheumatoid arthritis, Crohn's disease and leprosy during episodes of erythema nodosum leprosum (Rademacher *et al*, 1988; Filley *et al*, 1989), has led to the suggestion that raised agalactosyl IgG may be caused by a mycobacterial infection (Rademacher *et al*, 1989). Since a mycobacterial aetiology has been implicated in Takayasu's arteritis and sarcoidosis, as in rheumatoid arthritis and Crohn's disease, we measured the levels of agalactosyl IgG in serum samples of patients with these diseases.

3.2. Materials and Methods

3.2.1. Details of samples

There were 2 sets of serum samples from patients with sarcoidosis studied here. They were kindly provided by Dr O'Connor, Department of Medicine, University of Dublin, Ireland. The first set of samples contained 36 biopsy-proven sarcoid patients, of which 17 patients were female and 19 were male, with ages between 15 and 58 years (mean age 32 ± 8.9 yrs). For each patient, two bleeds were available. The initial bleeds were taken in the early course of the disease, and the other taken three years following initial sampling. According to the disease outcome on follow-up, patients were divided into four groups: Group 1, resolution of symptoms without corticosteroid (n=12); Group 2, resolution of symptoms following

treatment (n=6); Group 3, continued symptomatic disease with treatment (n=14); Group 4, unresolved but stable disease without treatment (n=4). The second set of serum samples included 73 patients with sarcoidosis. Of these, 37 were female patients and 36 were male. Their ages ranged from 24 to 76 years (mean age 41 ± 11.6 yrs). All serum samples of this set were taken within six weeks of the disease diagnosis. As controls, 36 serum samples from normal Irish individuals (17 female, 19 male, mean age = 34 ± 9.25) were also included.

The serum samples of Takayasu's arteritis and all the control samples were provided by Dr R Hernandez-Pando, Department of Pathology, University of Mexico City. Sera were obtained from 14 patients with Takayasu's arteritis whose age ranged from 11 to 50 years. Twelve patients were female and two were male. All of them began their disease when aged less than 30 years and first presented with fatigue or discomfort in the upper extremities and decreased pulses over one or both brachial arteries. All except one had vascular bruits over the subclavian arteries. The diagnosis was made according to clinical, laboratory and angiographic criteria described by the American College of Rheumatology for the classification of Takayasu's arteritis (Arend *et al*, 1990). Five of 14 patients were defined as having acute arteritis and nine of them as inactive disease. These patients were also checked for simultaneous occurrence of tuberculosis by microscopic examination of the culture of three sputum samples and at least one chest X-ray from each patient. Only one patient with inactive arteritis was found to be positive for TB with

an enlarged cervical lymph node. In addition, eight out of twelve patients showed positive skin test to PPD, two patients had a history of chronic contact with tuberculosis individuals, and none of the patients had been BCG vaccinated. Moreover, other symptoms, such as arthritis were found in four patients; fever in three and erythema nodosum in one. As positive controls, three serum samples from patients with pulmonary tuberculosis and three samples from patients with Crohn's disease were included. Negative controls came from five healthy individuals and five patients with Wegener's granulomatosis, another form of granulomatous arteritis. All patients with Wegener's granulomatosis were biopsied, and diagnosed according to the criteria of the American College of Rheumatology (Leavitt *et al*, 1990).

3.2.2. Measurement of %G(0) in sera

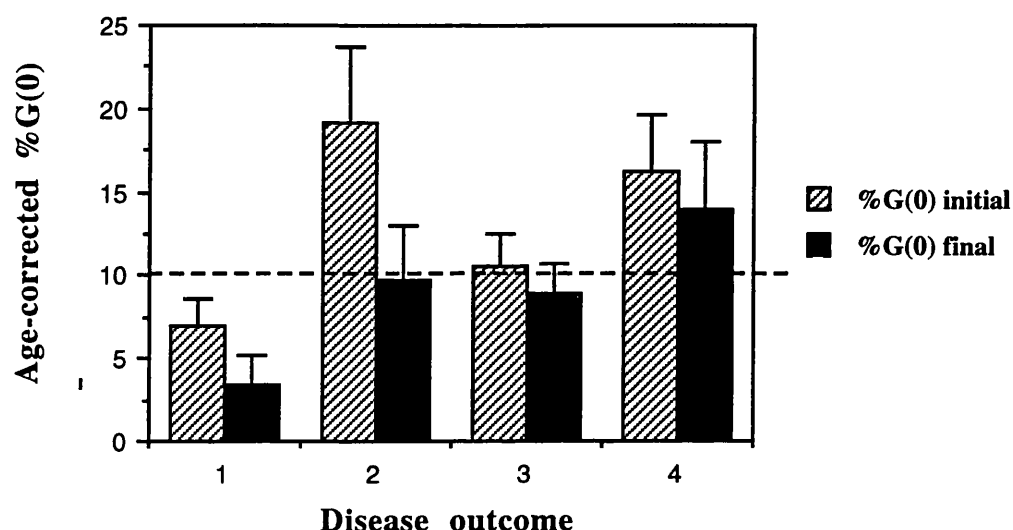
The %G(0) of all the samples studied here were tested blind using 'G(0) plate assay' as described in Chapter 2.

3.3. Results

3.3.1. %G(0) in sera from patients with sarcoidosis

In the first set of samples, the %G(0) were elevated in 16 out of 36 sarcoid patients at the time when the initial serum samples were taken. When these patients were divided into groups (Fig. 3.1), the mean initial %G(0) were abnormally higher in all other three groups except group 1 patients who showed spontaneous resolution of

Figure 3.1 Changes in serum %G(0) of patients with sarcoidosis after a three-year follow-up



Patients were divided into four groups according to the outcome of the disease. 1. resolution of symptoms without corticosteroid (n=12); 2. resolution of symptoms following treatment (n=6); 3. Continued symptomatic disease with treatment (n=14); 4. unresolved but stable disease without treatment (n=4). The percentage agalactosyl IgG were measured in the initial bleeds taken in the early course of the disease, and in the bleeds taken three years after the initial sampling. The results were expressed as the mean of the age-corrected percentage agalactosyl IgG plus standard deviation. The dotted line represents 2x SD above the normal age-corrected level of agalactosyl IgG.

symptoms. After a three year follow-up, the mean final %G(0) were significantly decreased in group 1 and 2 patients whose disease healed either spontaneously or following treatment ($p<0.05$). In contrast, no significant changes in %G(0) were observed in group 3 and 4 patients whose disease remained unresolved. In order to rule out the possible effect of corticosteroid, the %G(0) were measured in the second set of sarcoid samples which were taken at the time near diagnosis. As shown in Fig. 3.2, the %G(0) in sera from normal subjects all fell within 2x standard deviations of the mean value for normal donors of the same age; while the %G(0) in sera from patients with sarcoidosis were all elevated compared to that of the controls, and about 36 of the 73 patients whose %G(0) fell above 2x standard deviations, had abnormal high levels of agalactosyl IgG.

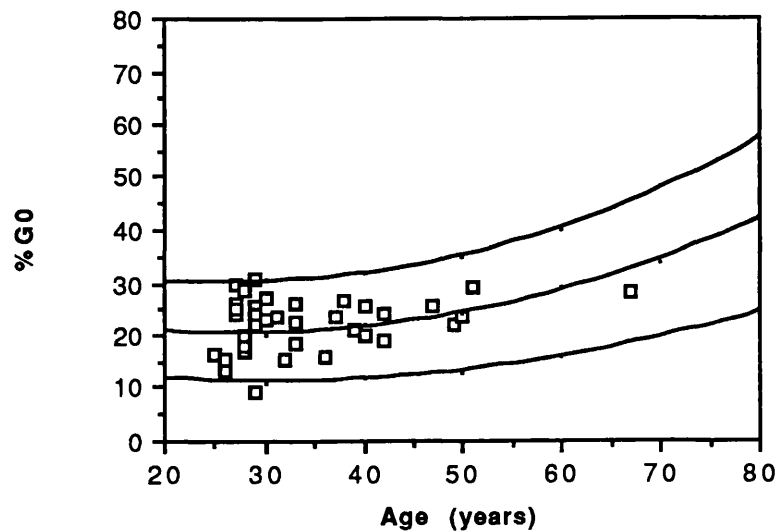
3.3.2. %G(0) in sera from patients with Takayasu's arteritis

The results of %G(0) in patients with Takayasu's arteritis are shown in Fig. 3.3a. A patient with inactive Takayasu's arteritis who also had active tuberculosis has been grouped with the other tuberculosis patients.

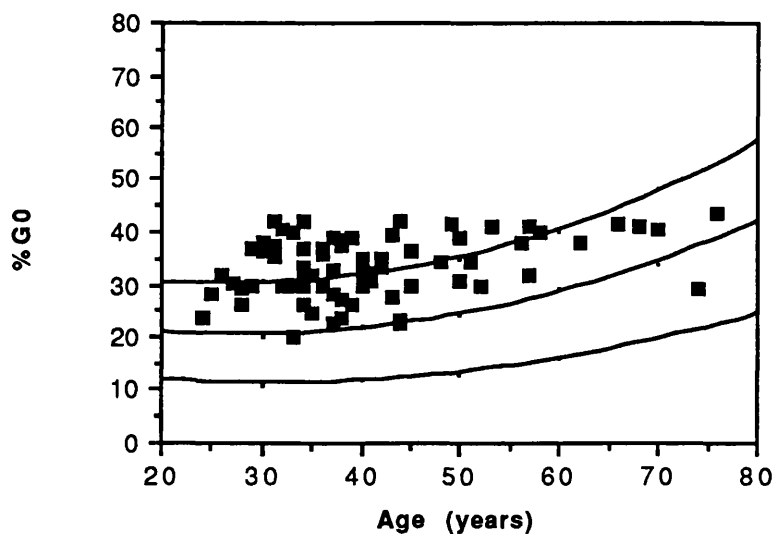
The %G(0) in sera from five patients with active Takayasu's arteritis ($40.3\pm 7.03\%$) was significantly higher than %G(0) in sera from normal donors ($25.2\pm 4.9\%$; $p=0.0043$, Student's t test, two tailed), or from patients with inactive Takayasu's arteritis ($24.4\pm 4.9\%$; $p=0.00059$). Moreover, the levels seen in these active patients were similar to those seen in the positive control sera from tuberculosis and Crohn's disease. In contrast, the %G(0) in sera from inactive

Figure 3.2 The %G(0) in sera from patients with sarcoidosis

The level of agalactosyl IgG in sera of control subjects

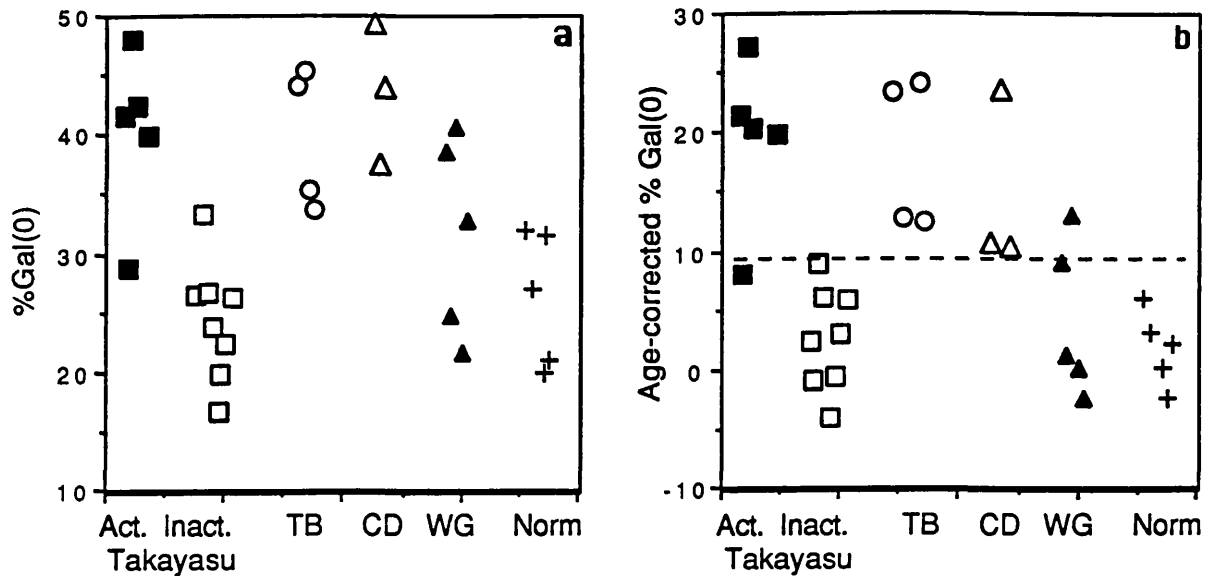


The level of agalactosyl IgG in sera of sarcoidosis patients



The percentage agalactosyl IgG in sera of 36 normal individuals and 73 patients with sarcoidosis were plotted against the age of the donors. The middle line indicates the mean percentage of agalactosyl IgG for normal donors of the same age. The area in between of the top and the bottom lines shows two standard deviations either side of the mean. About 36 patients with sarcoidosis fall outside the 2x SD.

Figure 3.3 The %G(0) in sera from patients with Takayasu's arteritis



a) The percentage agalactosyl IgG in sera from patients with active and inactive Takayasu's arteritis, or tuberculosis (TB), Crohn's disease (CD), Wegener's granulomatosis (WG) and normal donors (Norm). b) The same data corrected by subtracting the mean level of agalactosyl IgG found in normal European donors of the same age. The dotted line represents 2x SD above the normal age-corrected European level. The only patient with raised % agalactosyl IgG in the group with inactive arteritis also had active lymph-node tuberculosis and is plotted with the tuberculosis group.

Takayasu's arteritis resembled that seen in sera from normal donors, or patients with Wegener's granulomatosis ($31.7 \pm 8.3\%$), and was significantly lower than the levels in patients with Crohn's disease ($43.6 \pm 5.9\%$; $p=0.0004$) or tuberculosis ($39.7 \pm 5.9\%$; $p=0.0008$). After age-correction (Fig. 3.3b), the %G(0) values for all eight patients with inactive Takayasu's arteritis and four of the five patients with Wegener's granulomatosis fell within 2X standard deviations of the mean value for normal european donors of the same age. In contrast, the %G(0) values in sera of all positive control patients (tuberculosis and Crohn's) and four of the five patients with active Takayasu's arteritis fell above the 2X standard deviations.

3.4. Discussion

The present study demonstrated that: firstly, in patients with active Takayasu's arteritis the %G(0) were raised and were in the range previously seen only in RA, Crohn's disease and tuberculosis; secondly, the %G(0) were elevated in all 73 patients with sarcoidosis in comparison with that of the normal controls, and approximately half of the patients had abnormally high %G(0), in addition, there is a decline in %G(0) with the remission of sarcoidosis upon follow-up. The finding of raised agalactosyl IgG in patients with sarcoidosis was not consistent with the conclusion drawn from the previous study (Parekh *et al*, 1989). This could be due to a small number of patients analysed in the previous study. Moreover, the sarcoid patients analysed then may not have had active disease, so that the %G(0) were not high.

It is intriguing that agalactosyl IgG was only raised in a specific group of diseases, including rheumatoid arthritis, tuberculosis, Crohn's disease, sarcoidosis, leprosy during episodes of erythema nodosum leprosum (ENL) and Takayasu's arteritis. These diseases are either of mycobacterial origin, or have been linked tentatively with mycobacteria. Although an increase in %G(0) is not proof of a mycobacterial aetiology, it may imply a common aetiopathogenesis in these diseases. It was previously suggested that the increase in agalactosyl IgG was associated with a T cell-dependent tissue damaging pathology accompanied by cytokines release and an acute phase response (Rook *et al*, 1990c). This suggestion was based on the findings that, in Crohn's disease, raised %G(0) were found to correlate well with the levels of C-reactive protein (CRP) (Dube *et al*, 1990); in leprosy, a transient rise in %G(0) during episodes of ENL coincided with an acute phase response and with a rise in serum levels of free IL-2 receptors (Filley *et al*, 1989). It was emphasised that the %G(0) only rises when there is simultaneously an acute phase response and T cell-dependent tissue damage, and an acute phase response alone is not sufficient since the %G(0) was not raised in patients with acute rheumatic fever (Bahr *et al*, 1990) or in various viral infections (Rademacher *et al*, 1988). The finding in this study that the %G(0) was raised in patients with sarcoidosis does not seem to fit well with the above suggestion. Because sarcoidosis is a disease with T cell-dependent pathology but little tissue damage, and the levels of CRP are usually normal in patients with sarcoidosis (Hind *et al*, 1987). Thus raised %G(0) may only be associated with a T cell-mediated pathology with chronic release of cytokines. However, the type of

immunoregulatory disturbance resulting in the aberrant release of cytokines in these diseases is yet to be identified.

Although it was emphasised that raised agalactosyl IgG is not proof of a mycobacterial aetiology, the addition of Takayasu's arteritis to the exclusive list of diseases with raised agalactosyl IgG may be of particular interest. It was shown that in the same set of serum samples of Takayasu's arteritis which were also tested for the %G(0), the levels of antibodies to the purified 38-kD protein of *M. tuberculosis*, a highly specific mycobacterial antigen, and to the hsp65 protein of *M. leprae*, were significantly raised in 78% of the patients, and the levels were higher in those with active disease (Hernandez-Pando *et al*, in press). On the other hand, it is interesting to note that five patients with active Takayasu's arteritis all manifested autoimmune symptoms including arthritis and erythema nodosum. It is possible that in patients with Takayasu's arteritis mycobacterial hsp65 might elicit cross-reactive autoimmunity having as target the arterial wall. However, further studies of Takayasu's arteritis in this regard are needed.

A recent study suggested that a raised %G(0) is associated with potentially arthritogenic bacterial or viral infections, and is not necessarily an indicator of mycobacterial aetiology. In this study, the raised %G(0) were demonstrated in sheep and goats following infection with maedi visna virus and caprine arthritis encephalitis virus (CAEV), both of which can lead to inflammatory synovitis, as well as in the control group infected with *M. paratuberculosis* (McCulloch *et al*, in press). Moreover, in a family study including 8 patients with RA and their spouses and relatives, and another 13 pairs

of patients with RA and their spouses, it was found that 13 of the 21 spouses had raised %G(0), while most of the healthy first-degree relatives had normal %G(0) (Sumar *et al*, 1993). Similarly, the %G(0) were also raised in spouses of members of families with high incidence of autoimmune disease (Tomana *et al*, 1992), and spouses of patients with sarcoidosis (Fidler *et al*, submitted). These observations strongly suggested that an environmental factor is associated with raised %G(0), which could be an infection, but may not necessarily be of mycobacterial origin.

Chapter 4

Interleukin-6 and Agalactosyl IgG

4.1. Introduction

Interleukin-6 (IL-6) is a cytokine that has been found to have multiple biological activities, a number of which are involved in various aspects of immune and inflammatory responses (Kishimoto, 1989; Hirano *et al*, 1990; Akira *et al*, 1993). IL-6 can stimulate B cells to differentiate into plasma cells and to secrete immunoglobulin; stimulate liver cells to produce acute-phase proteins; enhance myeloma and hybridoma growth; support the proliferation of hematopoietic stem cells, and induce the differentiation and/or activation of T cells and macrophages.

IL-6 is produced by a number of different cell types, such as fibroblasts, monocytes/macrophages, endothelial cells, T and B lymphocytes, by exposure to a variety of extra-cellular stimuli (Ray *et al*, 1989). IL-6 is not produced under normal circumstances. However, following bacterial and viral infections, or tissue injuries in the host, IL-6 is rapidly and transiently expressed and participates in host defense. Other cytokines, such as interleukin-1 (IL-1) and tumour necrosis factor (TNF), can stimulate IL-6 production (Walther *et al*, 1988; Shalaby *et al*, 1989). In addition, it has been shown that IL-6 stimulates its own synthesis (Miyaura *et al*, 1989; Shabo *et al*, 1989).

The abnormal production of IL-6 was first found to be related to polyclonal B-cell activation with autoantibody production in patients with cardiac myxoma (Hirano *et al*, 1987). Since then, IL-6 has been suggested to be involved in the pathogenesis of a variety of diseases, such as autoimmune diseases, plasma cell neoplasias and glomerulonephritis.

In rheumatoid arthritis, elevated levels of IL-6 have been detected in the synovial fluid of affected joints and sera of the patients (Hirano *et al*, 1988; Houssiau *et al*, 1988). Significant correlations have been shown between the concentrations of synovial IL-6 and IgG, as well as between serum IL-6 activity and levels of a variety of acute-phase proteins such as C-reactive protein (CRP) (Houssiau *et al*, 1988; Hermann *et al*, 1989). IL-6 production has also been observed in type II collagen-induced arthritis in mice (Takai *et al*, 1989). Several other cytokines, such as IL-1 and TNF, are also present in synovial fluid. It is not known whether the abnormal expression of these cytokines is a primary event in the disease process, or a secondary consequence. IL-6 has been found to contribute to the proliferation of synovial lining cells, which results in the marked thickening of inflammatory tissue (pannus) in the joints of patients with RA (Firestein *et al*, 1990).

Abnormal production of IL-6 has also been observed in patients with Castleman's disease (Yoshizaki *et al*, 1989). This is a chronic disease with benign hyperplastic lymphadenopathy that is characterized by large lymph follicles with intervening sheets of plasma cells. The patients show hypergammaglobulinemia and

increase in acute-phase proteins and platelets. Clinical improvement and decrease in serum IL-6 levels were observed following resection of the hyperplastic lymph node, indicating that IL-6 is involved in the pathogenesis of the disease. In addition, IL-6 was found to be important for *in vivo* growth of murine plasmacytomas and human myelomas (Kawano *et al*, 1988). Multiple myeloma is a human B cell neoplasm characterized by accumulation, in the bone marrow, of plasma cells that secrete monoclonal immunoglobulins and by multiple osteolytic lesions. Plasmacytomas can be induced in BALB/c mice after intraperitoneal injection of pristane (2,6,10,14-tetramethylpentadecane, one of the non-metabolizable components of mineral or paraffin oil) (Potter and Boyce, 1962). These plasmacytomas were demonstrated to develop exclusively from cells in oil-induced granulomatous tissue, which were found to produce elevated concentrations of plasmacytoma growth factor(s), suggesting the importance of such growth factor(s) for the generation of plasmacytomas (Nordan and Potter, 1986). The mouse plasmacytoma growth factor was then found to be the murine homologue of human IL-6 (Hirano *et al*, 1986). A second consequence of injecting pristane into the peritoneal cavities of some strains of mice is the appearance of arthritic ankle and wrist joints after 100-200 days (Potter and Wax, 1981; Bedwell *et al*, 1987). It has been found that immunological mechanisms are involved in the development of pristane-induced arthritis because sublethally irradiated mice do not develop arthritis unless they are reconstituted by adoptive transfer of normal spleen cells (Bedwell *et al*, 1987). It is not known how pristane causes the onset of arthritis. However, it is possible that IL-6 may be involved,

since pristane induced granulomata are known to secrete IL-6. Raised agalactosyl IgG has been found in a specific group of diseases. However, the precise mechanism for such an increase in agalactosyl IgG is uncertain. It has been suggested that IL-6 may play an important role in controlling the production of agalactosyl IgG. It has been observed that the levels of agalactosyl IgG were strikingly raised in transgenic mice over-expressing the human IL-6 gene (Rook *et al*, 1991a). Moreover, several of the conditions where raised agalactosyl IgG were found, such as rheumatoid arthritis, Castleman's disease (Nakao *et al*, 1991), multiple myeloma (Nishiura *et al*, 1990), also have increased levels of IL-6.

In this study, we measured the levels of IL-6 and agalactosyl IgG in sera from patients with sarcoidosis, rheumatoid arthritis and pristane-injected mice, and sought for a correlation between the two parameters. Moreover, we also measured the levels of agalactosyl IgG in sera from mice that had been injected with recombinant IL-6.

4.2. Materials and Methods

4.2.1. Cell culture of B9 cells

B9 is a murine IL-6-dependent B cell hybridoma cell line. The B9 cells were maintained in RPMI 1640 medium (Imperial Laboratories) supplemented with 5% foetal bovine serum (FBS, Imperial Laboratories), 100U/ml penicillin, 100 µg/ml streptomycin and 25 pg/ml of IL-6, derived from the supernatant of MG63 osteosarcoma line (a kind gift from Dr A Meager, NIBSC, South

Mimms, Potters Bar, U. K.). The cells were cultured three times a week, and usually 1:6 split in fresh medium.

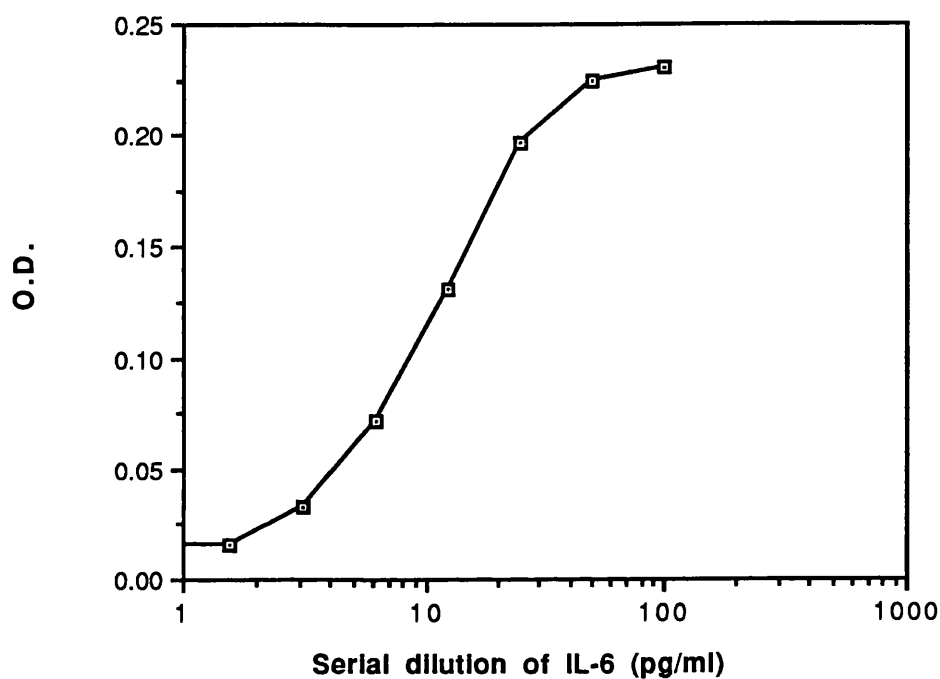
4.2.2. B9 cell bioassay

The IL-6 activity in serum samples were measured by this method. In each assay, a titration curve (Fig. 4.1) of standard IL-6 was included, ranging from 1 pg/ml to 100 pg/ml. The levels of IL-6 in samples were then read off from this curve. To assay human IL-6 activity, the serum samples were heat-inactivated in glass bijoux in a waterbath at 56°C for 30 minutes. The heat-inactivated sera were 3-fold serially diluted in 5% FBS/RPMI from 1:5 to 1:405. Then 50 µl of diluted sera per well were added to a 96-well flat bottomed microplate (Nunc) in duplicate.

The B9 cells used in the assay were in log-phase growth. The cells were washed three times with 5% FBS/RPMI by centrifugation at 1,300 rpm for 7 minutes to remove residual IL-6. After washing, the cells were resuspended in 5% FBS/RPMI at 5×10^4 /ml. Then 100 µl of the cell suspension were loaded to all the wells containing either 50 µl of diluted sera or titrated IL-6 standards.

After 3-4 days incubation at 37°C, 5% CO₂, the number of viable B9 cells was evaluated by an adaptation of the MTT colorimetric assay (Mosmann, 1983). Briefly, the cells in each well were pulsed with 7.5 µl of MTT (Sigma) solution at 5 mg/ml in PBS, and incubated at 37°C, 5% CO₂, for 40 minutes. Then 75 µl of 0.01M HCl/10% Triton (Sigma) solution was added to all wells to disrupt the

Figure 4.1 Titration curve of IL-6 standard in B9 bioassay



In each run of the B9 bioassay, a titration curve of IL-6 standard, ranging between 1.56-100 pg/ml, was included. The IL-6 titre of the unknown samples was read off the curve.

cells and dissolve the formazan precipitates. The optical density was then measured at 630 nm using an ELISA reader (DYNATECH, MR5000).

4.2.3. Measurement of agalactosyl IgG in mouse sera

The levels of agalactosyl IgG in murine sera were measured in this lab using the G(0) plate assay described in Chapter 2. In each plate, a limited series of standard murine sera with known %G(0) were included. Since the number of murine standards with known %G(0) was too low to form a calibration curve, the data for mouse sera are presented as the absorbance values (Rook *et al*, 1991).

4.2.4. Details of samples

The serum samples of patients with sarcoidosis were from one of the two sets of sarcoid samples studied in Chapter 3. This batch of samples included 73 patients, and the serum bleeds were collected at the time near diagnosis of the disease.

The 18 serum samples of patients with RA were chosen from the batch of Dutch patients with RA studied in Chapter 2.

The normal control sera were taken from the healthy individuals working in the lab.

The murine serum samples were provided by Dr S Thompson, Department of Pathology and Microbiology, University of Bristol. This study was carried out in collaboration with Dr Thompson, in which the titre of IL-6 was measured using B9 bioassay in his lab. The

level of agalactosyl IgG was measured using 'G(0) plate assay' in this lab. Briefly, 5 week old male DBA/1 mice were given a single intraperitoneal injection of pristane (Aldrich Chemical Co.) on day 0. Sera were obtained from 26 mice by aseptic cardiac puncture 160 days post pristane injection. At the same time, the peritoneal cavity was lavaged with 2.5 ml of sterile PBS. After aspiration and centrifugation at 1,400 g for 5 minutes, the supernatants were used as peritoneal exudate fluids (PEF). The recombinant IL-6 was injected intraperitoneally into male CBA/Ig^b mice aged between 8 and 16 weeks. One group (n=4/group) was given a single dose of IL-6 at 2×10^5 units whereas the other received repeated doses of IL-6 at 0, 3, 24 and 96 hours with 1×10^4 units. The control group was given PBS only on day 0. Sera were taken at day 0, 1, 4 and 7. All samples were stored at -20°C until used.

4.2.5. Statistical analysis

Student's t-test was used for the statistical comparisons along with Spearman's rank correlation test.

4.3. Results

4.3.1. Correlation of serum %G(0) with IL-6 titres in patients with sarcoidosis

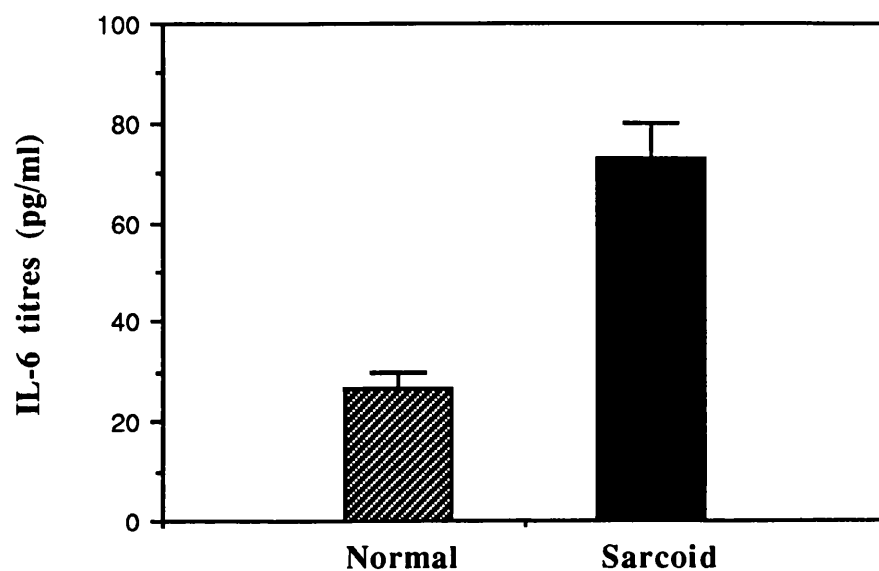
The IL-6 titres were measured in sera from 73 patients with sarcoidosis, which ranged from 15.5 pg/ml to 361 pg/ml. The levels of

IL-6 were found to be higher in 51 of the 73 patients with sarcoidosis when compared with those from normal individuals (19.5-41.9 pg/ml). The difference between the mean value of IL-6 levels in patients with sarcoidosis (73.1 ± 6.63 pg/ml) and those in normal subjects (26.8 ± 3.32 pg/ml) was statistically significant (Fig. 4.2, $p < 0.005$). The %G(0) in sera from these 73 sarcoid patients were measured by 'G(0) plate assay', which has been described in Chapter 3. It was shown that the %G(0) were increased in 36 of the 73 patients with sarcoidosis. In addition, the serum levels of IL-6 were found to be positively correlated with the %G(0) in those sarcoid patients (Fig. 4.3). Such a correlation is statistically significant ($n = 73$, $r = 0.301$, $p < 0.01$).

4.3.2. Correlation of serum %G(0) with IL-6 titres in patients with rheumatoid arthritis

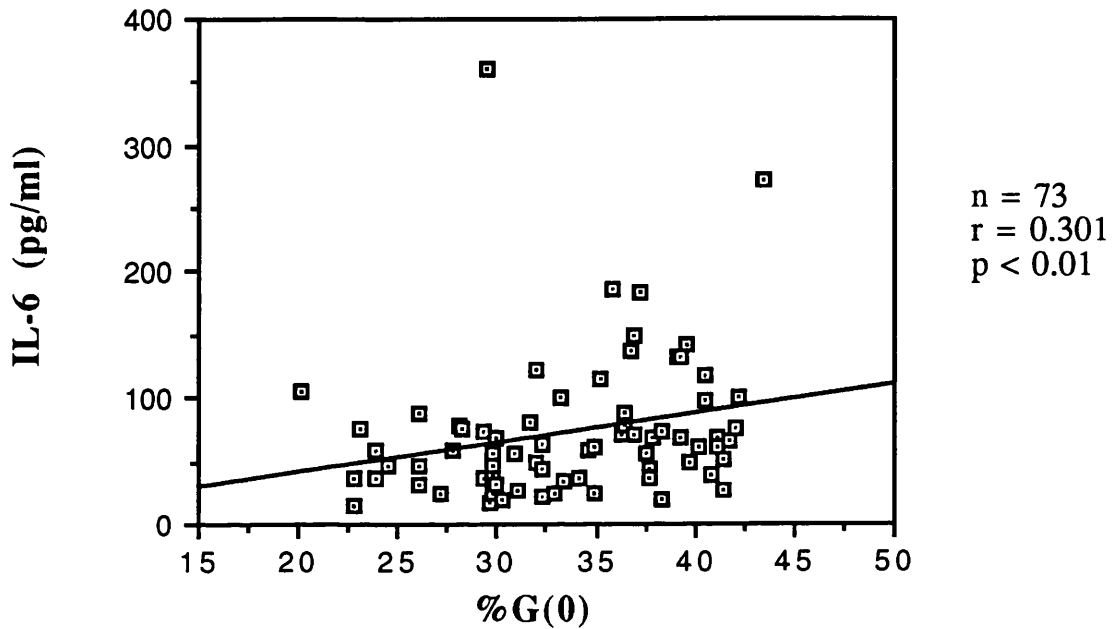
In this study, 18 patients with rheumatoid arthritis were analysed. The %G(0) in sera of these patients were measured using 'G(0) plate assay' as described in Chapter 2. It was found that the %G(0) were raised in 9 of the 18 patients ($46.72 \pm 4.3\%$), but remained normal in the other 9 patients ($21.5 \pm 2.6\%$). The IL-6 titres were found to be higher in 14 of the 18 patients with rheumatoid arthritis when compared with those in normal individuals (Fig. 4.4). The 9 patients with raised %G(0) all had a raised level of IL-6, while the other 9 patients with normal or lower %G(0), five of them also had a raised level of IL-6. No significant correlation has therefore been found between the serum levels of IL-6 and %G(0) in these 18 patients with rheumatoid arthritis (Fig. 4.5).

Figure 4.2 IL-6 titres in sera of patients with sarcoidosis



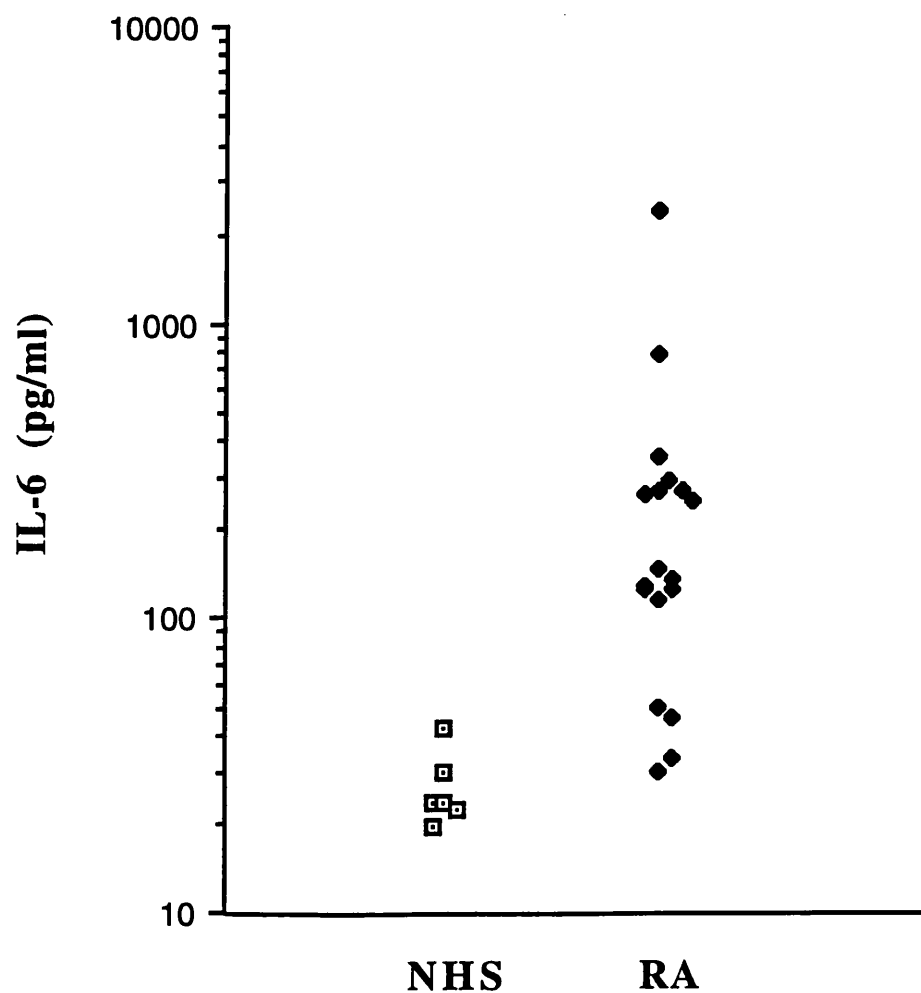
The IL-6 titres were measured in 36 normal individuals and 73 patients with sarcoidosis using the B9 bioassay. The mean titre of IL-6 in sera from normal individuals was compared with that from patients with sarcoidosis.

Figure 4.3 Correlation between IL-6 titres and %G(0) in sera from patients with sarcoidosis



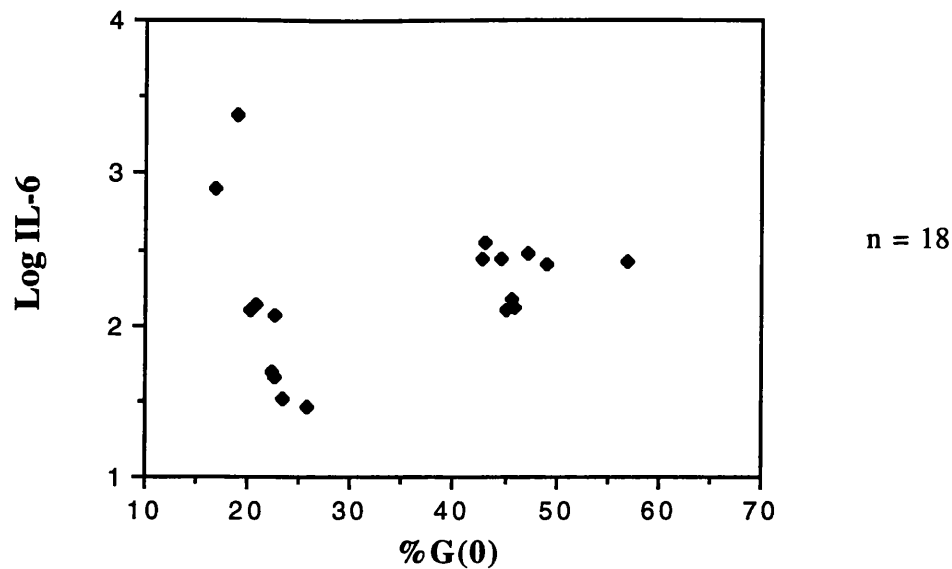
The serum IL-6 titres (vertical axis) in 73 patients with sarcoidosis were plotted against their serum percentage agalactosyl IgG (horizontal axis).

Figure 4.4 IL-6 titres in sera from patients with RA



The serum IL-6 titres in 18 patients with rheumatoid arthritis were compared with that in 6 normal individuals. IL-6 titres were increased in 14 of the 18 patients with rheumatoid arthritis.

Figure 4.5 Correlation between the serum levels of IL-6 and %G(0) in patients with RA



The serum IL-6 titres (log transformed) in 18 patients with rheumatoid arthritis were plotted against their percentage agalactosyl IgG .

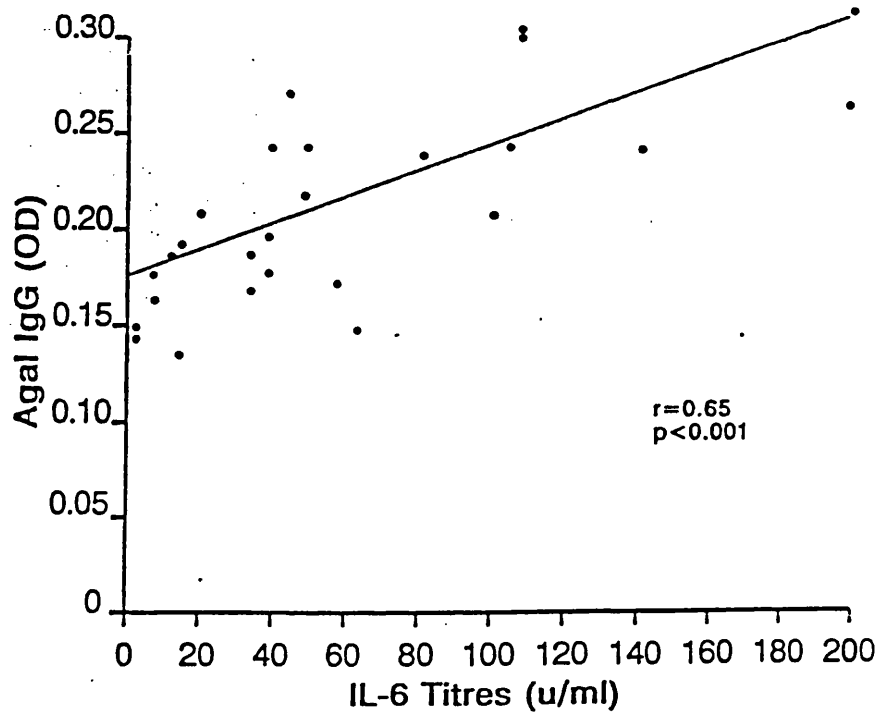
4.3.3. Correlation of serum agalactosyl IgG levels with IL-6 titres in pristane-injected DBA/1 mice

In 26 pristane-injected mice that may or may not have developed arthritis, the serum levels of agalactosyl IgG and IL-6 titres in sera or in peritoneal exudate fluid were measured. It was found that the serum levels of agalactosyl IgG positively correlated with IL-6 titres in peritoneal exudate fluid (Fig. 4.6, $r=0.65$, $p<0.001$), but not with IL-6 titres in sera of these pristane-injected DBA/1 mice.

4.3.4. Effect of recombinant IL-6 on the level of agalactosyl IgG in normal mice

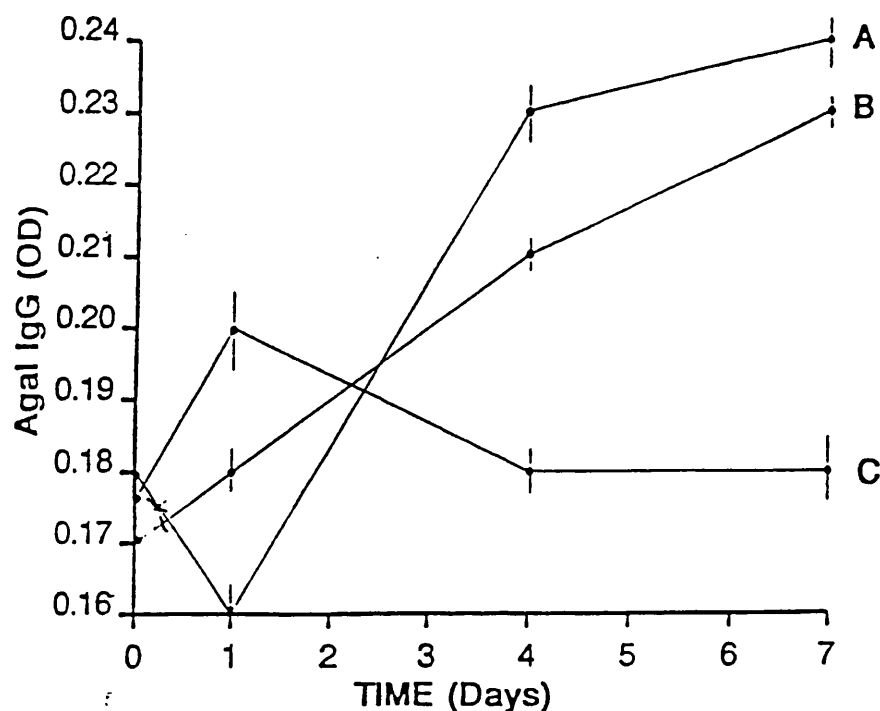
In this study, normal CBA/Ig^b mice were intraperitoneally injected with recombinant IL-6 either as one large dose or as smaller consecutive doses. The serum levels of agalactosyl IgG were measured at day 0, day 1, day 4 and day 7. As shown in Fig. 4.7, the levels of agalactosyl IgG were gradually increasing with time in mice that received repeated injections of recombinant IL-6; whereas in mice that were given only a single large dose of recombinant IL-6, the levels of agalactosyl IgG decreased slightly on day 1, then increased greatly on day 4 and day 7. The serum levels of agalactosyl IgG in mice injected with recombinant IL-6 were higher than that of PBS injected controls on day 4 and day 7. Such an increase in the serum levels of agalactosyl IgG in mice injected with IL-6 was found to be statistically significant by day 7 ($p<0.01$).

Figure 4.6 Correlation between serum agalactosyl IgG levels and PEF IL-6 levels (assayed by Dr. S. Thompson) in DBA/1 mice injected with pristane



The serum levels of agalactosyl IgG (vertical axis) in 26 DBA/1 mice injected with pristane were plotted against the IL-6 titres in peritoneal exudate fluid (horizontal axis) measured by Dr. S Thompson. All samples were obtained 160 days post pristane injection.

Figure 4.7 The development of agalactosyl IgG in CBA/Ig^b mice treated with rIL-6 by Dr. S Thompson



Three groups of CBA/Ig^b mice (n=4/group) were included in this experiment. Group A received a single dose of recombinant IL-6 of 2×10^5 units on day 0. Group B received repeated doses of recombinant IL-6 of 1×10^4 units at 0 and 3 hours and on day 1 and 4. The control group (C) received a single injection of PBS on day 0. The recombinant IL-6 and PBS were injected into mice intraperitoneally by Dr. S. Thompson. The levels of agalactosyl IgG were measured in sera of all 12 mice at various time points indicated.

4.4. Discussion

The present study was performed to elucidate the relationship between IL-6 and agalactosyl IgG. The levels of IL-6 and agalactosyl IgG were therefore measured in sera from patients with sarcoidosis and rheumatoid arthritis, as well as in sera and peritoneal fluid of mice injected with pristane. In addition, the serum levels of agalactosyl IgG were compared between mice injected with recombinant IL-6 and mice given PBS only.

In this study, most sarcoid patients (70%) had elevated serum levels of IL-6, and nearly half of the patients (49%) had raised serum levels of agalactosyl IgG. The mean value of IL-6 titres was significantly higher than those in normal subjects. Moreover, the serum levels of IL-6 were positively correlated with the serum levels of agalactosyl IgG in patients with sarcoidosis. Because increased release of IL-6 and TNF have been found from bronchoalveolar lavage cells of patients with active sarcoidosis (Homolka *et al*, 1993), it would be interesting to look at the levels of agalactosyl IgG in local lesions.

In rheumatoid arthritis, IL-6 titres were found to increase in sera from 14 out of 18 patients, and the levels of agalactosyl IgG were raised in 9 of the 18 patients. Although no significant correlation was found between the levels of agalactosyl IgG and IL-6, there was a trend that the levels of IL-6 were higher in patients with elevated levels of agalactosyl IgG, but lower in patients with normal levels of agalactosyl IgG (Fig. 4.5). The finding of the increase in serum IL-6 titres in patients with rheumatoid arthritis is consistent with the result

levels of agalactosyl IgG, but lower in patients with normal levels of agalactosyl IgG (Fig. 4.5). The finding of the increase in serum IL-6 titres in patients with rheumatoid arthritis is consistent with the result from a previous study (Houssiau *et al*, 1988). The serum IL-6 levels did not correlate with the levels of agalactosyl IgG, which could be due to the small number of samples studied here. On the other hand, treatment of rheumatoid arthritis with steroids could affect the levels of IL-6 (Akira *et al*, 1993).

In pristane-injected DBA/1 mice, the serum levels of agalactosyl IgG were found to correlate positively with IL-6 titres in peritoneal exudate fluids, but not with IL-6 titres in sera. The above result is irrespective of development of arthritis in pristane-injected mice. The finding that the serum levels of agalactosyl IgG were only significantly correlated with IL-6 titres in the peritoneal fluid, but not IL-6 titres in sera, may be due to the relatively short circulatory life of IL-6 (Castell *et al*, 1988). Binding of IL-6 to soluble receptors or inhibitors of IL-6 in sera, which could block the activity of IL-6 in the B9 bioassay, may also be responsible.

Direct intraperitoneal injection of recombinant IL-6 into normal mice could cause increased serum level of agalactosyl IgG. Thus this finding and all the above circumstantial evidence in this study taken together with the previous observation that IL-6 transgenic mice have strikingly raised levels of agalactosyl IgG are in support of the idea that raised agalactosyl IgG could be a consequence of increased levels of IL-6. However, it would appear that IL-6 is not the only factor involved in regulating the production of agalactosyl IgG since the relationship between the levels of agalactosyl IgG and IL-6 titres was

not apparent in some circumstances. For example, agalactosyl IgG was not raised in acute rheumatic fever (Bahr *et al*, 1990), during rejection episodes in heart or heart-lung transplant recipients (unpublished data), where IL-6 could be high. It would be interesting to look at the effects of other cytokines on the production of agalactosyl IgG. For instance, to measure the levels of agalactosyl IgG in the supernatant of a single B cell clone *in vitro* cultured in the presence of various cytokines. However, experiments of this kind were not undertaken in this study because they are included in a project of another PhD student in the Department of Immunology, which is currently in progress. In addition, IL-6 inhibitors could also be involved in regulating the production of agalactosyl IgG, which is discussed in the next chapter.

Chapter 5

The Specificity of an IL-6 Inhibitory Activity in Sera of Patients with Sarcoidosis

5.1. Introduction

5.1.1. The discovery of an IL-6 inhibitory activity in sera from patients with sarcoidosis

Sarcoidosis and tuberculosis are both characterized by the formation of T cell-dependent granulomata containing activated macrophages releasing large quantities of TNF (Rook *et al*, 1990b; Bachwich *et al*, 1986). But only patients with tuberculosis, not sarcoidosis, showed overt systemic upset, such as fever, weight loss and tissue damage both in the lesion and in tuberculin skin-test sites. These symptoms appear to be at least partly attributable to cytokines (Rook and Al-Attiyah, 1991b; Barnes *et al*, 1990). In an attempt to elucidate such an interesting difference between sarcoidosis and tuberculosis, previous authors from this department had discovered a TNF inhibitor present at high levels in sera from patients with sarcoidosis (Foley *et al*, 1990). However, this TNF inhibitor was also found at similar levels in patients with tuberculosis (Foley *et al*, 1990). Therefore, the minor systemic upset in patients with sarcoidosis could not be explained by the presence of TNF inhibitor.

Since the release of TNF and IL-1 is likely to be accompanied by the production of IL-6, both from the same macrophages and from other cells (Kishimoto, 1989), another striking difference between the two diseases is the nature of the acute phase response where IL-6 plays a major role. In tuberculosis, it was found that the level of C-reactive protein (CRP) was raised; whereas in sarcoidosis the CRP level was often close to normal (Hind *et al*, 1987). Because IL-6 is a major inducer of CRP synthesis in the liver (Li *et al*, 1990; Ganter *et al*, 1989), it was suspected that serum from patients with sarcoidosis might contain a selective inhibitor for IL-6, such that acute phase response, as reflected by the level of CRP, become deficient in sarcoidosis.

The second lead which was then thought to suggest a possible IL-6 inhibitor in patients with sarcoidosis came from a previous study showing that the levels of agalactosyl IgG were normal in most sarcoidosis patients, but high in patients with tuberculosis (Parekh *et al*, 1989), although we now know that the level of agalactosyl IgG can be abnormally high in sarcoidosis patients (see results of Chapter 3). The above suggestion was based on the fact that the levels of agalactosyl IgG were greatly elevated in transgenic mice over-expressing IL-6 gene (Rook *et al*, 1991a); however the finding that agalactosyl IgG levels were not high in sarcoidosis patients points to the presence of a possible IL-6 inhibitor in patients with sarcoidosis, but not in patients with tuberculosis.

Based on the above assumptions, Dr A. Verykaki carried out a series of studies during her one year visit in this department. She studied the serum levels of IL-6 using the proliferative response of an

IL-6-dependent B9 cell line, and sought evidence for an IL-6 inhibitor by quantitating inhibition of the proliferative response of B9 cells to IL-6 standards, in sera from normal controls (n=15), sarcoidosis (n=19) and tuberculosis (n=19). Among the nineteen sarcoidosis patients being tested, eight patients with active disease received steroid treatment. Since the B9 cell line is of murine origin, human sera are toxic to it. The toxicity was eliminated by heating the sera to 56°C for 30 minutes. It was found that the levels of IL-6 in heat-inactivated sera were higher in both patients with active sarcoidosis (0-584pg/ml) and patients with pulmonary tuberculosis at presentation (0-314pg/ml) than that of normal individuals. When the heat-inactivated sera from sarcoidosis and tuberculosis patients were assayed in the presence of 100 pg/ml of IL-6 standard, no inhibition of B9-cell proliferation in any case had been noted. However, when the serum samples were inactivated by zymosan and tested in the presence of 100 pg/ml of IL-6 standard, significant inhibition of B9-cell proliferation was observed in 16 out of 20 sarcoidosis patients, including those patients receiving steroids. In contrast, the inhibitory activity was not detectable in zymosan-inactivated sera of most tuberculosis patients at presentation, but levels rose progressively after initiation of treatment, and it was always demonstrable by 4-6 months before it began to fall. By that time, Dr Verykaki finished her visit and left. I then took over with an attempt to investigate the possible role of IL-6 inhibitor in regulating the production of agalactosyl IgG, and carried out the following experiments to find out the specificity of this heat-labile inhibitory activity.

5.1.2. Aim of this study

The present study was an extension of Dr Verykaki's work in order to investigate whether the heat-labile inhibitory activity detected is a specific inhibitor of IL-6. Thus, we looked at the effect of some inhibitor-containing sera of sarcoidosis patients on the proliferative responses of other cell types in comparison with those inhibitor-free sera of normal individuals.

5.2. Materials and Methods

5.2.1. Zymosan-inactivation of serum samples

Zymosan (Sigma) was boiled in normal saline for 45 minutes and then washed twice with sterile normal saline by centrifuging at 1,500 rpm for 10 minutes. The zymosan pellet was resuspended in sterile normal saline at 25 mg/ml. Each serum sample was mixed with zymosan at 6.25 mg/ml and incubated at 37°C for 30-45 minutes. The insoluble zymosan was then removed from each serum sample by spinning at 13,000 rpm for 10 minutes. The supernatant of each sample was collected and assayed afterwards.

The biological activity of IL-6, after zymosan treatment and heating at 56°C for 30 minutes, was tested as described below using B9-cell bioassay. A series of dilutions of human IL-6 standard (derived from the MG63 osteosarcoma line) was added to aliquots of a normal human serum known not to contain detectable IL-6. These

were then assayed after zymosan- and heat-inactivation. The results were compared with an IL-6 dose response curve performed simultaneously in the absence of human serum. As shown in Fig. 5.1, neither of the inactivation steps had any effect on the IL-6 dose response, confirming the stability of IL-6 in human serum at 56°C and after zymosan treatment.

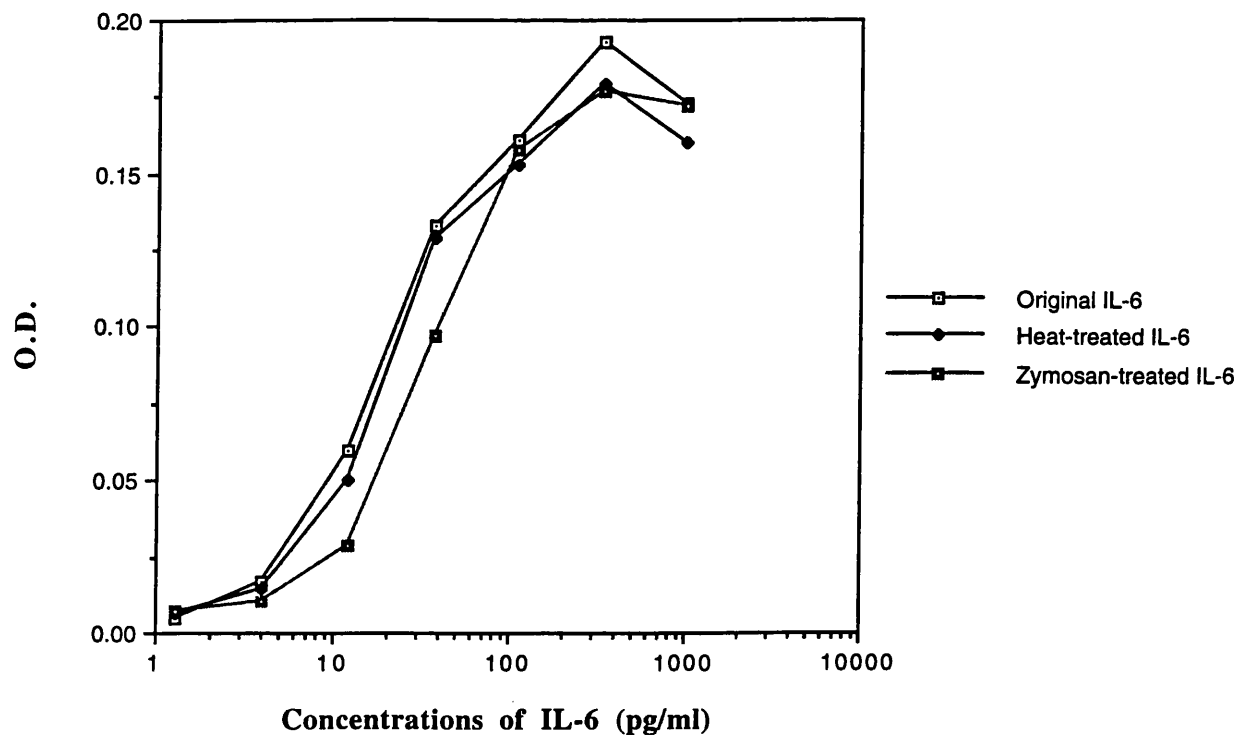
5.2.2. B9 cell bioassay for testing IL-6 inhibitory activity

The zymosan-inactivated serum samples were diluted in RPMI 1640 medium (Imperial Labs) containing 5% fetal bovine serum (FBS, Imperial Labs) and tested at a final dilution of 1:10 and 1:20 in the presence of 100pg/ml of IL-6 standard. In some experiments, in order to test the effect of TGF- β on the B9 cells, the above diluted serum samples were substituted with an equal volume of TGF- β which had been doubly diluted from 20 ng/ml, respectively.

B9 cells were maintained in RPMI 1640 medium containing 5% FBS and 25 pg/ml of IL-6. When used in the assay, cells were washed twice with RPMI 1640 only medium and seeded at 5×10^4 per well of a 96-well flat-bottomed tissue culture plate. In each plate, a titration curve of standard IL-6 was included, ranged between 1 to 100 pg/ml.

After 3-4 days of culture, the number of viable B9 cells was estimated by MTT colorimetric assay as described in Chapter 4. Any IL-6 inhibitory activity in the tested serum samples were then determined by comparing the viability of B9 cells, as reflected by optical densities, in the wells containing 100 pg/ml of IL-6 and tested sera with the viability of B9 cells in the wells containing 100 pg/ml of IL-6 alone.

Figure 5.1 Stability of serum IL-6 after heat and zymosan inactivation



The activity of human IL-6 standard was tested in the presence of a normal human serum known not to contain detectable IL-6, and that after heat- and zymosan-inactivation.

5.2.3. Other cell bioassays to check the specificity of the inhibitory activity in sera from patients with sarcoidosis

Serum samples from five sarcoidosis patients, which had been shown to have high levels of inhibitory activity for B9 cells, were tested for their influence on the proliferation of other four cell lines in comparison with serum samples from four normal individuals which did not have any inhibitory activity for B9 cells. Serum samples were zymosan-inactivated and assayed at 1:10 and 1:20 dilutions in duplicate. The four cell lines include: 1) B9.0, an IL-6-independent cell line; 2) IL-2-driven murine T cell blasts; 3) L929, a murine fibroblast line; 4) U937, a human promonocytic line. Moreover, another TGF- β -sensitive mink lung epithelial line, Mv-1-Lu, was used to show the activity of TGF- β at those concentrations which had been tested on the B9 cells.

1). An IL-6-independent cell line B9.0

B9.0 is a mutant from B9 cell line (a kind gift from Dr L. Aarden, Amsterdam, the Netherlands), which has become IL-6-independent. The B9.0 cells were maintained in RPMI 1640 medium containing 5% FBS. When used in the assay, the cells were resuspended at 10^5 /ml in fresh culture medium, and 75 μ l of the cell suspension was loaded into individual wells which contained 75 μ l of either the diluted serum samples or culture medium alone. After 4 days of culture at 37°C in a humidified 5% CO₂ incubator, the number of viable B9.0 cells was monitored by MTT colorimetric assay as

described in Chapter 4.

2). IL-2-driven murine T cell blasts

Two spleens from 6-8 weeks old CBA/BCIb mice were teased into single cell suspension in RPMI 1640 medium using forceps. The splenic cells were spun down at 1,000 rpm for 10 minutes, and then resuspended in 5 ml of haemolysis buffer (see APPENDICES) for 7 minutes to lyse the red blood cells. The cell suspension was then made up to 25 ml with RPMI 1640 medium and spun down. The cells were washed twice with RPMI 1640 medium containing 5% FBS by centrifuging at 1,000 rpm for 10 minutes. The viable cells were counted by trypan blue exclusion, and suspended at 10^7 /ml in RPMI 1640 medium containing 5% FBS and 8 μ g/ml of Concanavalin A (Sigma). After 48 hours culture, the stimulated lymphocytes were collected and seeded at 1.25×10^4 cells per well of a 96-well microtitre tray in RPMI 1640 medium containing 5% FBS. The serum samples were assayed in the presence of 100 U/ml of recombinant IL-2. A dose response curve of IL-2 ranging from 1 to 100 U/ml was also included. The assay was incubated at 37°C in a humidified CO₂ incubator for 48 hours before overnight pulsing with 1 μ Ci of [³H]-thymidine. The labelled cells were harvested onto a piece of glass microfibre filter paper (Whatman). The radioactivity was read out using liquid scintillation counter (LKB).

3). A murine fibroblast line L929

The L929 cells were cultured in RPMI 1640 medium (GIBCO) containing 100U/ml of penicillin, 100 μ g/ml of streptomycin, 1 μ g/ml

of fungizone, 1% of glutamine (GIBCO) and 5% of FCS (GIBCO). When used in the assay or to subculture, the confluent adherent cells were stripped off the bottom of the culture flasks by incubating with EDTA/trypsin solution (see APPENDICES) for about 5 minutes at room temperature. The detached cells were spun down at 1,100 rpm for 4 minutes, and resuspended at 3×10^5 cells/ml in fresh culture medium. Then 75 μ l of the cell suspension was dispensed into individual wells of a 96-well flat-bottomed microtitre tray. The cells were allowed to settle for about 1 hour at 37°C in a humidified CO₂ incubator before loading with 75 μ l of the diluted testing samples. A medium alone control was included. After 24 hours of culture, the cells were pulsed with 1 μ Ci of [³H]-thymidine overnight. Then the medium in individual wells was sucked away, and the cells were detached from the plate by incubation with EDTA/trypsin solution and harvested onto a piece of glass microfibre filter paper. The radioactivity was estimated by liquid scintillation counting.

4). A human promonocytic line U937

The U937 cells were cultured in RPMI 1640 medium (GIBCO) containing 10% fetal calf serum (FCS, GIBCO), 5×10^{-5} M β -mercaptoethanol (GIBCO), 100 U/ml of penicillin and 100 μ g/ml of streptomycin. Cells were split twice a week and subcultured at 2.5×10^5 /ml in fresh medium. When used in the assay, 75 μ l of the cell suspension at 4×10^4 /ml were loaded into each well of a 96-well microtitre tray which contained 75 μ l of the diluted serum samples. The assay was terminated after 72 hours incubation. The proliferation response was also estimated by [³H]-thymidine incorporation as

described above.

5). A TGF- β -sensitive mink lung epithelial line Mv-1-Lu

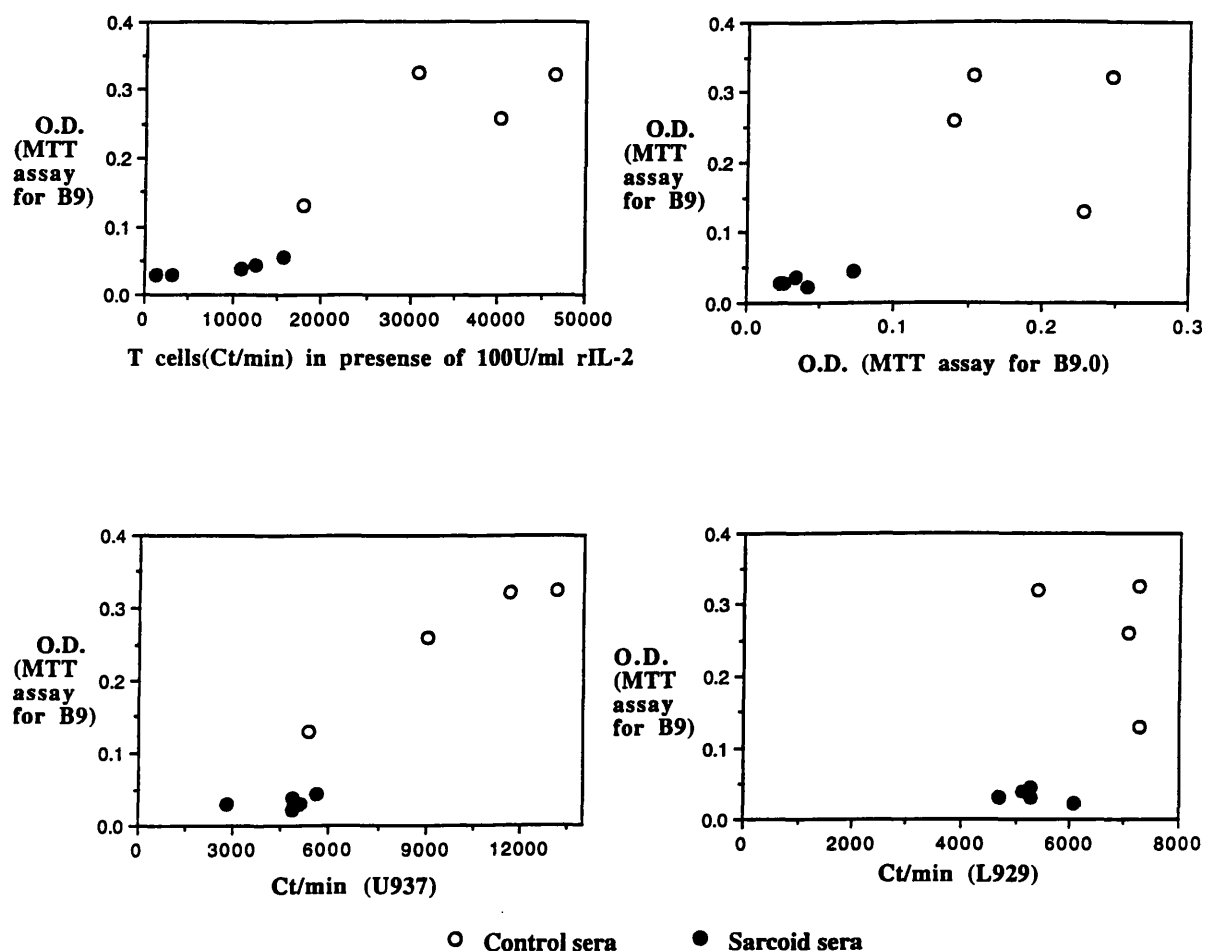
Mv-1-Lu (Meager, 1991) is a mink lung epithelial line which is extremely sensitive to the growth inhibitory activity by TGF- β . It has been shown that the Mv-1-Lu cells can be completely halted from proliferating by TGF- β at as low as 1 ng/ml. It has therefore been recommended as the most suitable cell line for measuring the activity of TGF- β . This cell line was kindly provided by Dr T. Meager (NIBSC, Potters Bar, South Mimms, U.K.).

The Mv-1-Lu cells were maintained in RPMI 1640 medium (GIBCO) containing 7% FCS, 100 U/ml of penicillin and 100 μ g/ml of streptomycin. When confluent, the cells were harvested from the culture flasks by EDTA/trypsin treatment, and spun down at 1,100 rpm for 4 minutes, then subcultured in fresh medium. In the assay, the cells were seeded at 10^4 per well of a 96-well microtitre tray, in which 150 μ l of serially diluted recombinant TGF- β had been added. The assay was incubated at 37°C in a humidified 5% CO₂ incubator for 3 days. The number of viable cells was determined by MTT colorimetric assay.

5.3. Results

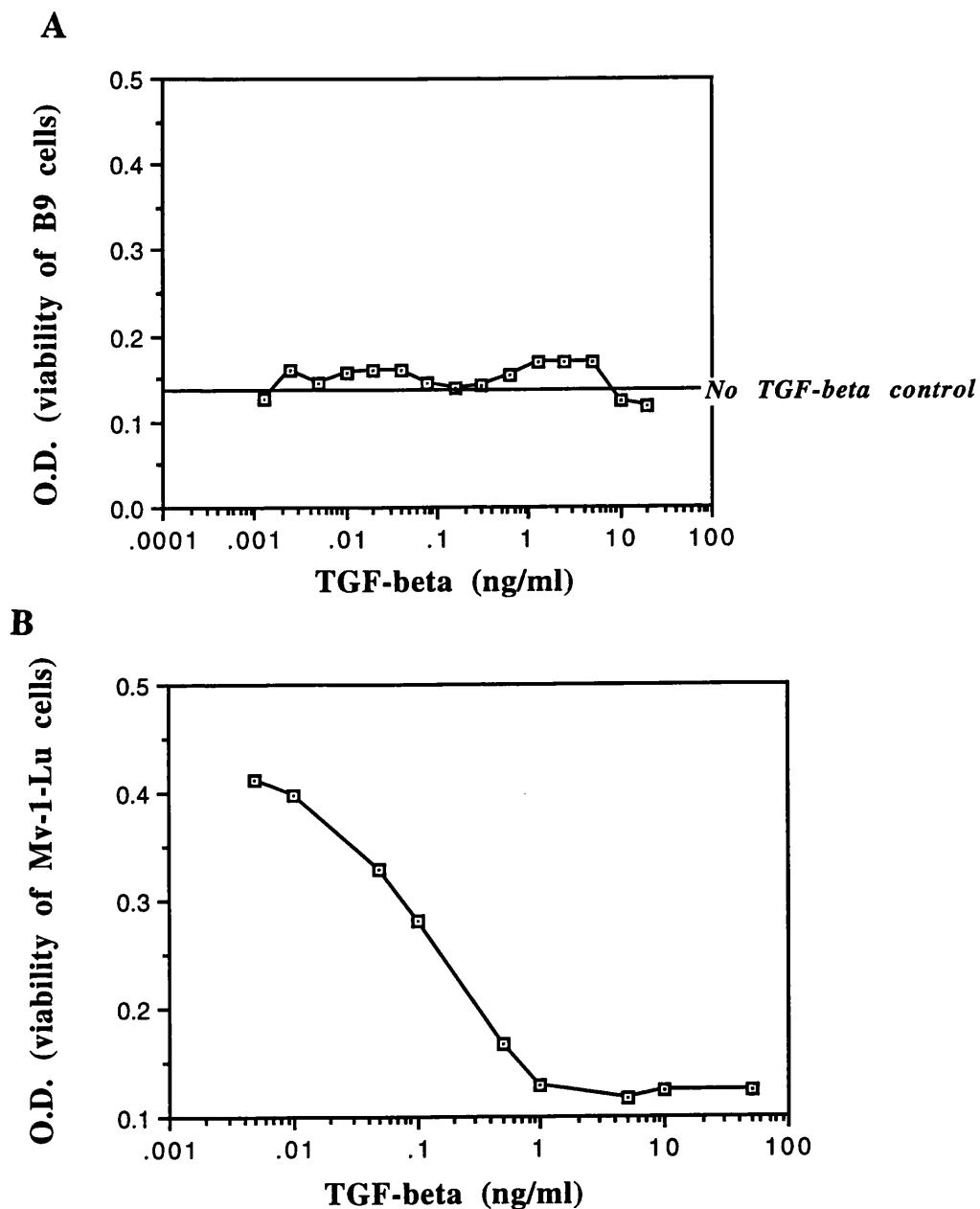
In order to find out whether the heat-labile inhibitory activity detected is a specific inhibitor of IL-6, we tested sera of five sarcoidosis patients known to have the inhibitory activity, for their ability to inhibit the growth of four other cell lines. These are an IL-6-independent variant of B9 (B9.0), IL-2-driven murine T cell blasts, murine fibroblasts (L929) and human promonocytic cells (U937). In each case, the correlation between inhibition of B9 cells, and inhibition of the other cell types was strong (Fig. 5.2). In particular, the IL-2-driven murine T cell blasts and the IL-6-independent variant (B9.0), whose growth were suppressed almost as strongly as that of the B9 cells by the inhibitor-containing sera of sarcoidosis patients in contrast to the sera from normal individuals. The inhibition was clearly demonstrated at 1:10 serum dilution. Therefore, the heat-labile inhibitory activity appears to be a non-specific inhibitor of cell growth. Since TGF- β is known to inhibit the growth of many cell types (Barnard *et al*, 1990), the sensitivity of B9 cells to this cytokine was tested. No effect on B9-cell growth was seen at concentrations of TGF- β as high as 20 ng/ml (Fig. 5.3A), whereas the growth of Mv-1-Lv was completely inhibited at 1 ng/ml of TGF- β (Fig. 5.3B). This meant that the nil effect of TGF- β on the growth of B9 cells was not due to the insufficient biological activity of the cytokine. Thus, the cell growth inhibitory activity in sera from sarcoidosis patients was not TGF- β .

Figure 5.2 Inhibition of cell proliferation by zymosan-inactivated sera from patients with sarcoidosis



The inhibition of IL-6-driven proliferation (vertical axis), by zymosan-inactivated sera from 4 normal individuals and from 5 sarcoidosis patients, was shown in association with the inhibition of cell proliferation of four other cell types (horizontal axis).

Figure 5.3 Failure of TGF- β to inhibit the proliferation of B9 cells



The proliferation of B9 cells were tested in the presence of TGF- β at various concentrations between 0.001 ng/ml and 20 ng/ml (A). No inhibition was shown at concentrations 20-40x greater than required to completely inhibit the proliferation of the Mv-1-Lu cell line (B).

5.4. Discussion

This study was carried out to elucidate the specificity of a heat-labile IL-6 inhibitor found in sera of patients with sarcoidosis. The results showed that this heat-labile inhibitory activity is not a specific IL-6 inhibitor, but rather an inhibitor of cell proliferation. This inhibitory activity may have gone unnoticed in the past because human sera have been routinely heat-inactivated before being used in the B9 bioassays. The inhibition was not due to excess IL-6 because it can be present in patient's sera which do not contain detectable levels of the cytokine. In addition, it seemed also not to be the effect of steroids because the inhibitory activity was present in sera of sarcoidosis patients not receiving this treatment, and in sera of tuberculosis patients who were treated with non-steroid drugs. Moreover, pilot experiments done by Dr Verykaki showed that the concentrations of steroids (prednisolone and prednisone) needed to achieve complete inhibition of the response of B9 cells to IL-6 were 10^{-5}M and 10^{-4}M , respectively (Dr A. Verykaki personal communication). To achieve such concentrations in our experiments, serum steroid concentrations of 10^{-4} to 10^{-3} would be required (sera were tested at 1:10 dilution), and these were 100-1000 fold greater than the peak levels seen during steroid therapy (Pickup, 1979). On the other hand, this study showed that the inhibition of the response of B9 cells to IL-6 was not the action of $\text{TGF}\beta$ either, although $\text{TGF}\beta$ had been reported to inhibit the proliferation of a variety of cell types including those of epithelial, endothelial and lymphoid origin

(Barnard *et al*, 1990).

Several other workers have reported human inhibitors of cell proliferation (Jegasothy and Battles, 1981; Malkovsky *et al*, 1983; Aune and Pierce, 1981; Green *et al*, 1981; Wilkins *et al*, 1983; Santoli *et al*, 1986). For instance, there was a report of an activity which inhibited the proliferation of L929 cells, but was not heat-labile (Jegasothy and Battles, 1981). Others described a heat-labile factor, but it had no inhibitory effect on U937 or L929 (Santoli *et al*, 1986). Similarly there was another heat-labile inhibitor of DNA synthesis, but its effect was confined to B and T lymphocytes (Wilkins *et al*, 1983). Thus none of these reports closely match the present findings. Further characterization of this inhibitory activity would be interesting. However, since this inhibitory activity is not a specific inhibitor of IL-6, further work in evaluating its role in regulating the production of agalactosyl IgG was not continued in this study.

Chapter 6

The Biological Properties of Agalactosyl IgG

6.1. Introduction

Agalactosyl IgG is a glycoform of IgG which contains no terminal galactose residues on the carbohydrate chains located in the CH2 domains (Parekh *et al*, 1985). Increased levels of agalactosyl IgG have been found before the onset of rheumatoid arthritis (Schrohenholer *et al*, 1991), and also been shown to correlate with the disease activity (Parekh *et al*, 1988; Young *et al*, 1991). On the other hand, decreased levels of agalactosyl IgG have been found to correlate with pregnancy-induced remission of rheumatoid arthritis (Rook *et al*, 1991c). Moreover, the level of agalactosyl IgG rises rapidly after parturition as symptoms of rheumatoid arthritis return. These findings seem to imply that agalactosyl IgG may be involved in the pathogenesis of rheumatoid arthritis, or may play a regulatory role in this disease (Rademacher, 1991). It has been suggested that the lack of terminal galactose in agalactosyl IgG could lead to a conformational change in the CH2 domain (Parekh *et al*, 1985). Moreover, human IgG degalactosylated with β -galactosidase showed a decreased binding to Fc γ R expressed on a monocytic cell line U937 (Tsuchiya *et al*, 1989). Therefore, it is possible that agalactosyl IgG may also show a reduced binding to Fc γ RII which is the sole Fc receptor expressed on B cells, and fail to exert the negative feedback on B cells via Fc

receptors which is characteristic of normal IgG, and may instead provide a positive signal (Rademacher, 1991).

Receptors for the Fc region of IgG, Fc γ R, mediate important regulatory and effector functions within the immune system. Currently, three classes of Fc γ R have been identified on human (h) leukocytes: hFc γ RI (CD64), hFc γ RII (CD32), and hFc γ RIII (CD16). They can be distinguished on the basis of their molecular weight, cellular distribution, affinity and specificity for IgG isotypes, and recognition by monoclonal antibodies (mAbs) (Ravetch and Kinet, 1991; van de Winkel and Anderson, 1991; van de Winkel and Capel, 1993).

The most widely distributed Fc γ R class is hFc γ RII, which is present on B cells, monocytes/macrophages, granulocytes, platelets, placental endothelium, and Langerhans cells. The hFc γ RII is a 40 kD glycoprotein with very low affinity for monomeric IgG and interacts with IgG only in complexed or polymeric form. Multiple isoforms of hFc γ RII have been found, which are highly homologous in their extracellular and transmembrane regions but exhibit significant divergence in their cytoplasmic domains (Stuart *et al*, 1987 & 1989; Brooks *et al*, 1989). The hFc γ RII isoforms are designated hFc γ RIIa1, soluble hFc γ RIIa2, hFc γ RIIb1, hFc γ RIIb2, hFc γ RIIb3 and hFc γ RIIc. These isoforms are encoded by three different genes, Fc γ RIIA, Fc γ RIIB, and Fc γ RIIC, all localized on chromosome 1 (Qiu *et al*, 1990; Warmerdam *et al*, 1993a).

The hFc γ RIIA gene shows allelic variation, which results in two allotypes, namely (high responder: HR) hFc γ RIIa-HR and (low responder: LR) hFc γ RIIa-LR. These two allotypes were originally

distinguished by different reactivities with mouse IgG1 complexes. It was shown that monocytes from different individuals interact either strongly or weakly with mouse IgG1 complexes, referred to as high or low responder individuals (van de Winkel and Anderson, 1991). Subsequently, these allelic variants have also been found to differ in their ability to bind human IgG2 complexes. The hFcγRIIa-LR allotype can effectively bind human IgG2 complexes in contrast to the hFcRIIa-HR allotype (Warmerdam *et al*, 1991). In addition, a monoclonal anti-FcγRII antibody (mAb 41H16) has been identified, which has a unique specificity for an epitope expressed on the hFcRIIa-HR allotype (Gosselin *et al*, 1990). The molecular basis of this functional polymorphism between hFcγRIIa-HR and hFcγRIIa-LR has been studied. It was revealed that one amino acid difference at position 137, arginine (HR) and histidine (LR), is critical for the polymorphic reactivity with mouse IgG1, human IgG2 complexes, as well as mAb 41H16 binding (Warmerdam *et al*, 1990 & 1991; Clark *et al*, 1991). Recently, a polymorphism was also noted in hFcγRIIb1, in which a single nucleotide difference was found to result in one amino acid change in the cytoplasmic tail of hFcγRIIb1. This variant of hFcγRIIb1 is designated as hFcγRIIb1* (Warmerdam *et al*, 1992b). Interestingly, hFcγRIIb1* can also be detected by mAb 41H16.

The exact cell-type distribution of these various isoforms of hFcγRII is not very clear. It is generally considered that the hFcγRIIa isoform is solely expressed on myeloid cells, whereas hFcγRIIb isoforms seem to be preferentially expressed on B lymphocytes (Ravetch and Kinet, 1991). However, hFcγRIIa expression has recently been detected in circulating human B lymphocytes and B cell

lines (Mantzioris *et al*, 1993; van den Herik-Oudijk *et al*, 1993). It was shown that pre-B cell lines expressed exclusively the hFcγRIIa isoform, whereas the more mature B cell lines expressed hFcγRIIa, hFcγRIIb1 and hFcγRIIb2 isoforms (van den Herik-Oudijk *et al*, 1993).

The recent application of transfecting FcR negative cell lines with cDNA clones encoding different receptors has provided a useful tool to study the biological function of a specific FcR class (van de Winkel and Anderson, 1991). The hFcγRIIa isoform has been shown to be capable of mediating phagocytosis of antibody-opsonized erythrocytes (EA) upon transfection into COS, 3T6, or P388D1 cell lines (Indik *et al*, 1991; Tuijnman *et al*, 1992; Odin *et al*, 1991), whereas the hFcγRIIb1* molecule has been found to be inactive in EA phagocytosis upon transfection into 3T6 cells (Tuijnman *et al*, 1992). Recently, different FcγRII isoforms have been transfected into mouse IIA1.6 B cell line (van den Herik-Oudijk *et al*, 1993). It was found that hFcγRIIb1, particularly hFcγRIIb1* molecules have more effective capping ability than hFcγRIIa or hFcγRIIb2 isoforms. Moreover, in contrast to hFcγRIIa isoforms, the hFcγRIIb1, hFcγRIIb2 and hFcγRIIb1* molecules were found capable of down-regulating the Ca²⁺ influx on cross-linking with cell surface IgG.

The Fc receptors (FcγRII) on B lymphocytes have been well described in their ability to regulate B-cell activation by membrane immunoglobulins (mIgs). Cross-linking of mIg by multivalent antigen or by F(ab')₂ fragments of anti-mIg antibody can result in B-cell proliferation and differentiation. However, if mIg is cross-linked with surface FcγR through intact anti-mIg antibodies or antibody-antigen

complexes, a dominant inhibitory signal is conferred to prevent the B-cell activation (Phillips and Parker, 1984; Sinclair and Panoskaltsis, 1987). This Fc-dependent regulation has been suggested to play a role in the induction of tolerance by haptenated IgG (Borel, 1976; Waldschmidt *et al*, 1983), and in controlling the B-cell repertoire by anti-idiotypes (Sinclair and Panoskaltsis, 1987).

In this study, we employed two experimental models to compare the ability of galactosyl and agalactosyl IgG to bind to cells bearing Fc γ RII *in vitro* and to regulate the hapten-specific tolerance *in vivo*. Firstly, we carried out EA-rosette assays, using human Fc γ RII transfected cell lines and IgG monoclonal anti-Rh D antibodies which were treated with and without β -galactosidase. Secondly, we reconstructed the tolerance model induced by haptenated isologous IgG in mice (Borel, 1976), and then looked at the effect of agalactosyl haptenated IgG on the anti-hapten responses by plaque forming assay.

6.2. Materials and Methods

6.2.1. EA-rosette formation *in vitro*

6.2.1.1. Human monoclonal anti-D antibodies

Monoclonal anti-D antibodies are specific for the Rh D antigen on human red blood cells. Two of these human monoclonal anti-D antibodies were used in the present study, namely BRAD-3 and BRAD-5. They are kindly provided by Dr Kumpel, International

Blood Reference Lab, Bristol, and were produced from Epstein-Barr virus transformed B lymphoblastoid cell lines derived from three hyperimmune donors (Kumpel *et al*, 1989). BRAD-3 is of IgG3 isotype with kappa light chains, which is also known as 1A3-3. BRAD-5 is of IgG1 isotype with kappa light chains, which is also known as 1A11. Human IgG were purified from serum-free culture supernatant (Iscove's modified DMEM) containing anti-D monoclonal antibodies using Protein G sepharose column (Pharmacia). BRAD-3 and BRAD-5 have been shown to have the glycosylation profiles that were similar to normal serum IgG, although the %G(0) of these two antibodies, as determined by biochemical analysis and G(0) immunoassay, were less than 10% (Kumpel *et al*, 1995).

6.2.1.2. Preparation of agalactosyl IgG anti-D monoclonal antibodies

The method used here has been described previously (Tsuchiya *et al*, 1989). Briefly, 350 µg aliquots of each IgG anti-D antibody were treated with 10 mU of β-galactosidase from *Streptococcus* strain 6646K (ICN Flow) in 100 µl of 100 mM citrate phosphate buffer pH 6.0. Digests were incubated at 37°C for 18 hours. The reaction was terminated by adding 100 µl of 20 mM Na₂HPO₄. The IgG anti-D antibodies were repurified from the digests on a Protein G sepharose column (Pharmacia). This enzyme treatment resulted in an increase in %G(0) of BRAD-5 from 3.6% to 30%, and of BRAD-3 from about 2% to 20%, as determined using G(0) plate assay as described in Chapter 2. In the G(0) assay, the initial step was slightly altered, that is, Protein G was used instead of Protein A to coat the plate, as Protein

A has a very low affinity of binding to human IgG3 (Antibodies, a laboratory manual, edited by Harlow and Lane).

6.2.1.3. Human red blood cells

The red cells used in this study were from two Rh D positive (RhD⁺) donors. One donor was of blood group O -D/-D-, whose red cells had been found to express high numbers of D antigens (approximately 100,000 D sites/cell). The other donor was an ordinary A RhD⁺ (homozygous for D) with about 40,000 D sites/cell. The -D- red blood cells were kindly provided by Dr Kumpel, International Blood Group Reference Lab, Bristol. As a negative control, red cells from a Rh D negative donor were also included in the study.

6.2.1.4. Sensitization of red cells with anti-D monoclonal antibodies

To sensitize the red cells, 100 µl of anti-D antibodies, which were doubly diluted from 40 µg/ml in 5% FCS/RPMI, were mixed with an equal volume of 5% red cell suspensions in round-bottomed glass tubes. The mixture was incubated at 37°C for 2 hours, with agitation 3-4 times during this period. Red cells were then washed 3 times with 5% FCS/PBS (pH 7.3) by centrifuging at 2,000 rpm for 5 minutes. The supernatant was removed and the final red cell pellet was either gently resuspended in 5% FCS/RPMI to be used in the rosetting assay, or lysed with solubilizing buffer to quantify the cell-bound IgG.

6.2.1.5. Solubilization of the sensitized red cells

To solubilize the sensitized and washed red cells, 80-90 μ l of solubilizing buffer [5% v/v Triton X-100 with 2 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM EDTA in Ca^{2+} and Mg^{2+} free PBS] was added to the above red cell pellet, and was agitated for 5 minutes at room temperature. The lysates were diluted at 1/5, 1/10, 1/50 and 1/100 in 5% FCS/RPMI. Then a IgG capture ELISA was carried out to determine the amount of IgG anti-D antibodies bound to each red cell.

6.2.1.6. Quantitation of red cell-bound IgG anti-D antibody

The method used here to quantify the number of IgG anti-D antibody molecules bound to each red cell was a simple non-isotopic ELISA (Kumpel, 1990a).

The 96-well flat-bottomed ELISA plates were coated with 50 μ l/well of 1/200 diluted goat anti-human IgG (Sigma) in 0.05 M carbonate buffer pH 9.6 for 15 minutes at 37°C in a shaking incubator. Then 100 μ l of PBS/BSA/T20 was added to each well to block the non-specific binding sites for 30 minutes at 37°C in a shaking incubator. The plates were washed three times with PBS/T20. The diluted red cell lysates as well as human IgG standard (0.016-1 μ g/ml, Dako) were loaded into the plate in triplicate, 50 μ l/well, and incubated for 1 hour at 37°C in a shaking incubator. The plates were washed again three times with PBS/T20, followed by addition of 50

μl/well of peroxidase-conjugated goat anti-human IgG (Sigma) which is diluted 1/500 in PBS/BSA/T20. The plates were incubated at 37°C in a shaking incubator for one hour, followed by washing three times with PBS/T20. The colour was developed by using 50 μl/well of ABTS substrate solution (see APPENDICES). The reaction was stopped by addition of 50 μl/well of NaF solution (see APPENDICES). The optical densities were read using an ELISA reader at 630/490 nm wavelengths. The concentrations (μg/ml) of IgG in the lysates were obtained from IgG standard titration curve (Fig. 6.1).

The number of IgG molecules bound to each red cell was calculated using the following formula:

The molecular weight of IgG is taken as 160,000. The Avogadro constant is 6.022×10^{23} . Therefore, 1 mg IgG contain 375×10^{10} molecules ($6.022 \times 10^{23} / 160,000 \times 10^6$), respectively.

The number of IgG molecules/cell = xy, where,

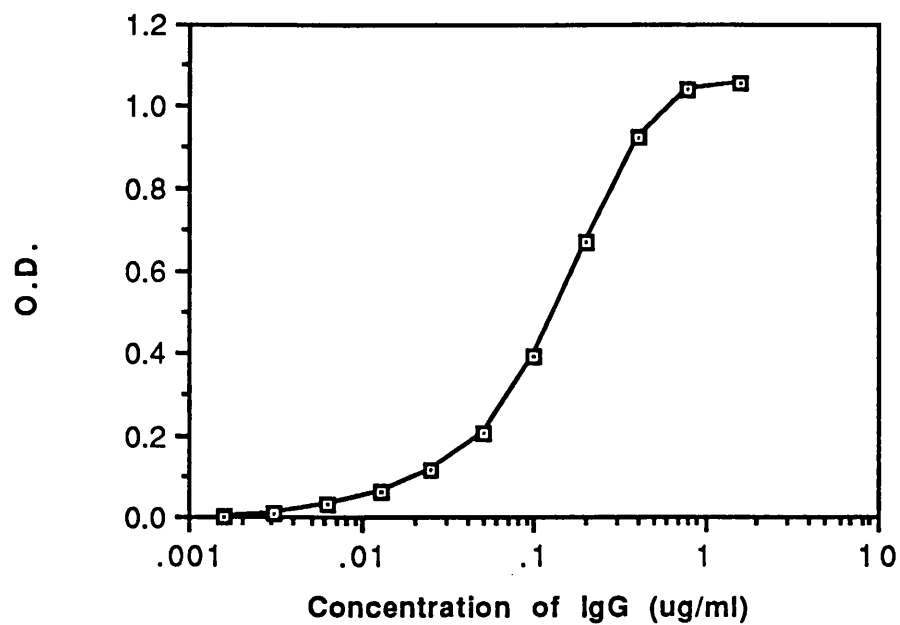
x = concentration of IgG in lysate (μg/ml)

y = $\frac{\text{molecules of IgG}/\mu\text{g}}{\text{number of red cells/ml}}$

6.2.1.7. Cell culture of human FcγRII transfectant cell lines

These cell lines were of mouse fibroblasts (3T6) transfected with human FcγRII cDNAs, namely pPW3-2B3 (hFcγRIIa-LR), pcD16.2-9A4 (hFcγRIIa-HR) and B1-14-2B2 (hFcγRIIb1*), respectively (Warmerdam *et al*, 1990 & 1993). They were kindly provided by Dr Van de Winkel from the Department of Immunology, University Hospital, Utrecht, the Netherlands.

Figure 6.1 Titration curve of human IgG standard



Human IgG standard, ranging from 0.016 $\mu\text{g/ml}$ and 1 $\mu\text{g/ml}$, was included in each run of ELISA. The concentration of IgG ($\mu\text{g/ml}$) of unknown samples was read off the curve.

The two hFcγRIIa expressing cell lines (pPW3-2B3 and pcD16.2-9A4) were grown in RPMI 1640 medium containing 5% FCS (Imperial). The cell line transfected with hFcγRIIb1* (B1-14-2B2) was maintained in RPMI 1640 medium containing 5%FCS, mycophenolic acid (20 μg/ml), thymidine (5 μg/ml), xanthine (10 μg/ml), hypoxanthine (15 μg/ml), deoxycytidine (2.3 μg/ml) and aminopterin (0.2 μg/ml). The cells were cultured in a 37°C, 5% CO₂ incubator. The culture medium was replenished every 3 to 4 days. The cells were isolated from the culture flasks by incubation with trypsin/EDTA solution (see APPENDICES) for 5 minutes at room temperature, and washed once by centrifuging at 1,100 rpm for 4 minutes. An aliquot of the cell pellet was resuspended in 15 ml of fresh medium.

6.2.1.8. Indirect immunofluorescence

The expression of hFcγRII on transfected cell lines was assayed by indirect immunofluorescence, using specific anti-FcγRII monoclonal antibodies and FITC-labelled F(ab')₂ fraction of goat anti-mouse IgG antiserum (DAKO). Fluorescence intensities were measured by a FACScan flow cytometer (Becton & Dickinson). Two monoclonal anti-FcγRII antibodies were used, namely 41H16 and IV.3 (murine IgG2a and IgG2b isotypes), which were obtained from Professor Lydyard's lab, Dept of Immunology, Middlesex Medical School, London.

Cells were harvested from culture flasks by incubation with

trypsin/EDTA solution for 5 minutes at room temperature, which were then spun down at 1,100 rpm for 4 minutes. The supernatant was decanted and the cell pellet was resuspended in fresh medium to 2×10^6 /ml. Then 100 μ l of this cell suspension was loaded into each well of a 96-well U-bottomed plate, and spun for 5 minutes at 1,000 rpm. The supernatant in the wells was sucked off carefully using a pump. The cell pellet in each well was resuspended in 20 μ l of either fresh medium alone or medium containing optimal amount of anti-Fc γ RII monoclonal antibodies. A control antibody P3 (murine IgG1 isotype), which was known not to bind to any cell surface components, was also included. After 45 minutes incubation on ice, the cells were washed three times by addition of 200 μ l/well PBS containing 1%BSA and 0.01% sodium azide, and centrifuged at 1,000 rpm for 5 minutes. The cells were then incubated with 20 μ l/well of FITC-conjugated F(ab')₂ fragment of goat anti-mouse IgG antiserum, which was diluted 1:20 in PBS containing 1%BSA and 0.01% sodium azide, for another 45 minutes on ice. After final three washes, the cells were fixed with 200 μ l/well of 2% paraformaldehyde. Fluorescence intensities from 5,000 cells were measured using a FACScan flow cytometer.

6.2.1.9. Pronase treatment of Fc γ RII transfected cells

The transfected cells were harvested from culture flasks using EDTA/trypsin treatment as described above, and resuspended at 2×10^6 /ml in either RPMI 1640 medium alone or medium containing pronase (Sigma) at different concentrations ranging from 5 μ g/ml to

500 µg/ml. After 30 minutes incubation at 37°C, 5% CO₂, cells were washed three times with RPMI 1640 medium by centrifuging at 1,100 rpm for 4 minutes. The final cell pellet was resuspended in 25 ml of 5% FCS/RPMI medium at 1×10^4 /ml, and then used in the rosetting assay.

6.2.1.10. EA-rosette formation

EA-rosette assay is one of the well-established methods to study IgG-Fc receptor interactions (Kerbel and Elliott, 1983). Rosettes were formed by mixing antibody coated erythrocytes (EA) with cells bearing Fc receptors. In the present study, the indicator cells are red cells sensitized with anti-D monoclonal antibodies, the effector cells are the transfectant cells expressing FcγRII. Briefly, the transfectant cells were harvested from culture flasks and resuspended at 1×10^4 cells/ml in 5% FCS/RPMI medium. This cell suspension was then aliquotted into 24-well tissue culture plates (Nunc), 1 ml per well. After 2 hours incubation at 37°C, 5% CO₂, cells were stuck to the bottom of the plates, the upper medium was carefully decanted and replaced with 0.5 ml of the suspension of sensitized red cells at 1.5×10^7 cells/ml in 5% FCS/RPMI medium. The plates were kept at 4°C for 90 minutes. The wells were gently rinsed twice with cold PBS. The percentage of rosettes formed was quantified microscopically. In each sample, 200 cells (in the central area of the wells) were examined and scored positive when three or more red cells were bound. Unsensitized red cells and sensitized Rh D negative red cells were included as controls, and never showed any binding. In some

experiments, the rosettes were fixed with 2.5% glutaraldehyde (in PBS) for 10 minutes at room temperature. After two washes with PBS, the wells were covered with 0.4% trypan blue solution for 5 minutes at room temperature. The dye was then rinsed off with distilled water.

6.2.2. Induction of tolerance by haptenated isologous IgG *in vivo*

6.2.2.1. Conjugation of DNFB to KLH

This method was described in 'Practical Immunology' (Hudson and Hay, third edition). Briefly, keyhole limpet haemocyanin (KLH, Sigma) was dissolved in 1 M NaHCO₃ at a concentration of 10 mg/ml. To 10 ml of this suspension, 0.5 ml of 2,4-dinitro-1-fluorobenzene (DNFB, Sigma) was added. The mixture was stirred vigorously at 37°C for 45 minutes in the dark. The precipitates were spun down. The dinitrophenyl (DNP)-KLH conjugates were separated from the free DNFB on a sephadex G-25 column (Pharmacia). The optical densities of the conjugates were measured at 360nm and 278nm wavelengths using a spectrophotometer. The substitution ratio of DNP-KLH was calculated using the following conversion:

DNP: at 360 nm, absorbance of 1.0 (1 cm cuvette) is equivalent to 0.067 mmol DNP.

KLH: at 278 nm, absorbance of 1.0 (1 cm cuvette) is equivalent to 0.00018 mmol KLH.

In this calculation, an average molecular weight of KLH was assumed at 3×10^6 . A background optical density reading of KLH alone at 360

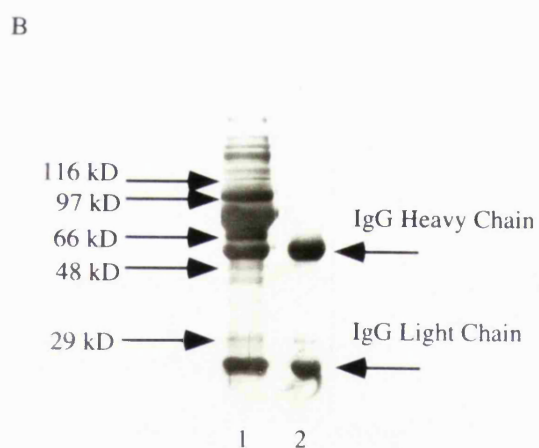
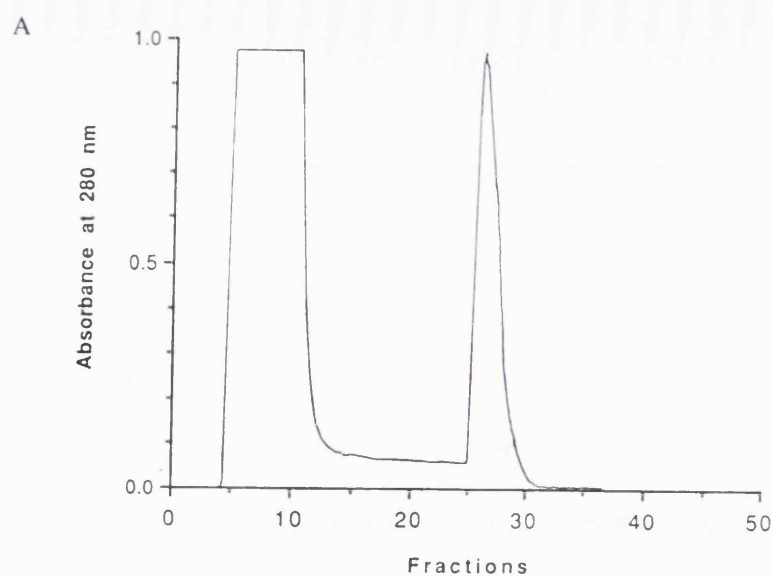
nm was subtracted from the optical density reading at this wavelength of DNP-KLH conjugates. This preparation has resulted in DNP-KLH conjugates of 200-240 moles of DNP per mole of KLH.

6.2.2.2. Affinity purification of IgG using protein G sepharose column

A protein G sepharose column (Pharmacia) was equilibrated with 30 ml of binding buffer (20 mM sodium phosphate, pH 7.0). The samples were diluted 1:1 with binding buffer and applied on to the column. When the samples had drained into the gel bed, another 30 ml of the binding buffer were added onto the column to wash away the unbound protein. The bound IgG was eluted by filling the column with about 15 ml of elution buffer (100 mM glycine-HCl, pH 2.7). Every 1 ml of the eluted fraction was collected into each of the prepared tubes containing 80 μ l of neutralizing buffer (1 M Tris-HCl, pH 9.0) through a fraction collector (LKB). The purification process was monitored through a detector/chart recorder system (LKB). The column was re-equilibrated with binding buffer, then filled with 20% ethanol and stored at 4°C. The fractions containing eluted IgG were pooled together and dialysed against PBS. The purified IgG was stored at -20°C for future use.

The typical purification profile is shown in Fig. 6.2A. Fractions 25-30 were collected, which contained the purified IgG. When the purity of IgG was examined by SDS-PAGE, two clean bands were demonstrated (Fig. 6.2B), which represented the heavy and light chains of IgG.

Fig. 6.2 The IgG purification profile



IgG was purified from sera using a protein G column (panel A). Fractions 5–15 were the unbound protein, and the fractions 25–30 eluted from the column were the purified IgG. The purity of the eluate was checked by 10% SDS-PAGE (panel B). Lane 1: protein marker; Lane 2: the purified IgG (fractions 25–30).

6.2.2.3. Conjugation of TNBS to IgG

The method has been described in 'Practical Immunology' (Hudson and Hay, third edition). IgG was purified from pooled sera of pregnant C57 black mice using a protein G sepharose column. The buffer of the purified IgG was exchanged from PBS to 0.15 M K₂CO₃ using sephadex G-25 column. To 4 mg of IgG, 1 mg of 2,4,6-trinitrophenol-sulphonic acid (TNBS, Sigma) was added. The mixture was incubated at 4°C overnight in the dark. The trinitrophenol (TNP)-IgG conjugates were isolated from free TNBS by running through a sephadex G-25 column. The substitution ratio of TNP-IgG was calculated as follows:

X - The molar concentration of TNP:

$$\frac{\text{Optical density of the conjugate at 360 nm wavelength}}{14,400 \text{ (the molar absorptivity of TNP at 360 nm)}}$$

Y - The molar concentration of IgG:

$$\frac{\text{Concentration of the conjugate (mg/ml)}}{150,000 \text{ (the molecular weight of IgG)}}$$

The TNP/IgG ratio = X / Y

Using this method, the molar substitution of the TNP-IgG conjugates were found to be 7 moles TNP per mole of IgG.

6.2.2.4. Preparation of agalactosyl TNP-IgG

The above TNP₇-IgG conjugates were depleted of terminal galactose by β-galactosidase treatment as described in section 6.2.1.2. Briefly, the concentration of TNP₇-IgG conjugates was adjusted to 4 mg/ml. The buffer of the conjugate was exchanged from PBS to CPB

(0.1 M citrate phosphate buffer, pH 6.0) by dialysis. Then 0.1 unit of β -galactosidase from *streptococcus* strain 6646K was added. The mixture was incubated at 37°C for 18 hours. The enzyme was removed from the digests using the protein-G sepharose column. As a control, another aliquot of the conjugates went through the same procedure except that no β -galactosidase enzyme was added. After enzyme treatment, the molar substitution of the TNP-IgG conjugates was re-examined and found to be the same as the non-enzyme treated control. On the other hand, the galactosylation status of the TNP-IgG conjugates was monitored before and after enzyme treatment by G(0) plate assay as described in Chapter 2. The level of G(0) (expressed as optical densities) of the non-enzyme-treated TNP-IgG conjugates was 0.230; whereas the level of G(0) of the enzyme-treated TNP-IgG conjugates was raised to 0.516, respectively.

6.2.2.5. Immunization of mice

Male C57 black mice aged 16 weeks were bred in our animal facility. 12 mice were used in this experiment, 4 mice in each group. To prime the mice, a single i.v. injection (0.2 ml) of 0.2 mg of TNP-IgG conjugate (β -galactosidase treated and non-treated) or PBS alone was given. Immediately thereafter, all mice (include control mice) were challenged with an i.p. injection of 0.2 mg of DNP-KLH conjugates emulsified in complete Freund's adjuvant (see APPENDICES). The mice were sacrificed five days later to obtain the spleens.

6.2.2.6. TNP sensitization of sheep red blood cells (SRBC)

Sheep red blood cells in Alsevers solution (TCS) were washed twice with PBS by spinning at 2,000 rpm for 5 minutes. Then 1 ml of packed SRBC were added dropwise into 7 ml of 0.28 M cacodylate buffer (see APPENDICES) containing 50 μ l of liquid TNBS at 950 mg/ml. The mixture was stirred for 10 minutes at room temperature in the dark, and the reaction was stopped by addition of chilled PBS. The TNP-coupled SRBC were washed with cold RPMI 1640 medium until no free (yellow) TNP was visible in the supernatant. Finally, the concentration of TNP sensitized SRBC was adjusted to 20% (v/v) in RPMI 1640 medium and stored at 4°C.

6.2.2.7. Hemolytic plaque assay

This method is a modification of the Jerne haemolytic plaque assay, and described in Practical Immunology (Hudson and Hay, third edition).

Glass microscopy slides were used to prepare the assay chambers. The slides were washed thoroughly with a free rinsing detergent, and soaked overnight in absolute ethanol, then air dried. A sandwich chamber was made by coupling two glass slides together with a double-sided sealer tape stuck firmly to the short edges and the centre row in parallel with the short edges of the slides.

The spleens were removed from the injected mice after 5 days. A single cell suspension of each spleen was prepared by teasing with

forceps. The splenic cell suspension was washed once with RPMI 1640 at 4°C by centrifuging at 1,000 rpm for 15 minutes. The cell pellet from each spleen was resuspended in 1 ml of RPMI 1640 medium as a stock. In each well of a micro-titre tray, 50 µl of RPMI 1640 medium, 25 µl of 20% TNP sensitized SRBC suspension, 25 µl of neat guinea-pig serum absorbed with SRBC, and 100 µl of splenic cell suspension appropriately diluted from the stock were mixed together. This mixture was then loaded into an assay chamber along the long edges using a Pasteur pipette. When the chambers were filled, the long edges of the chamber were sealed with a pre-melted paraffin wax-petroleum jelly mixture. These chambers were then incubated at 37°C without fan for about 20-30 minutes, and taken out as soon as plaques were clearly visible to the naked eye. The number of plaques formed in each chamber were counted using a low-power binocular microscope, which was then multiplied by the dilution factor to obtain the number of plaque-forming cells (PFC) per spleen. Results were expressed as the geometric mean of four individual values.

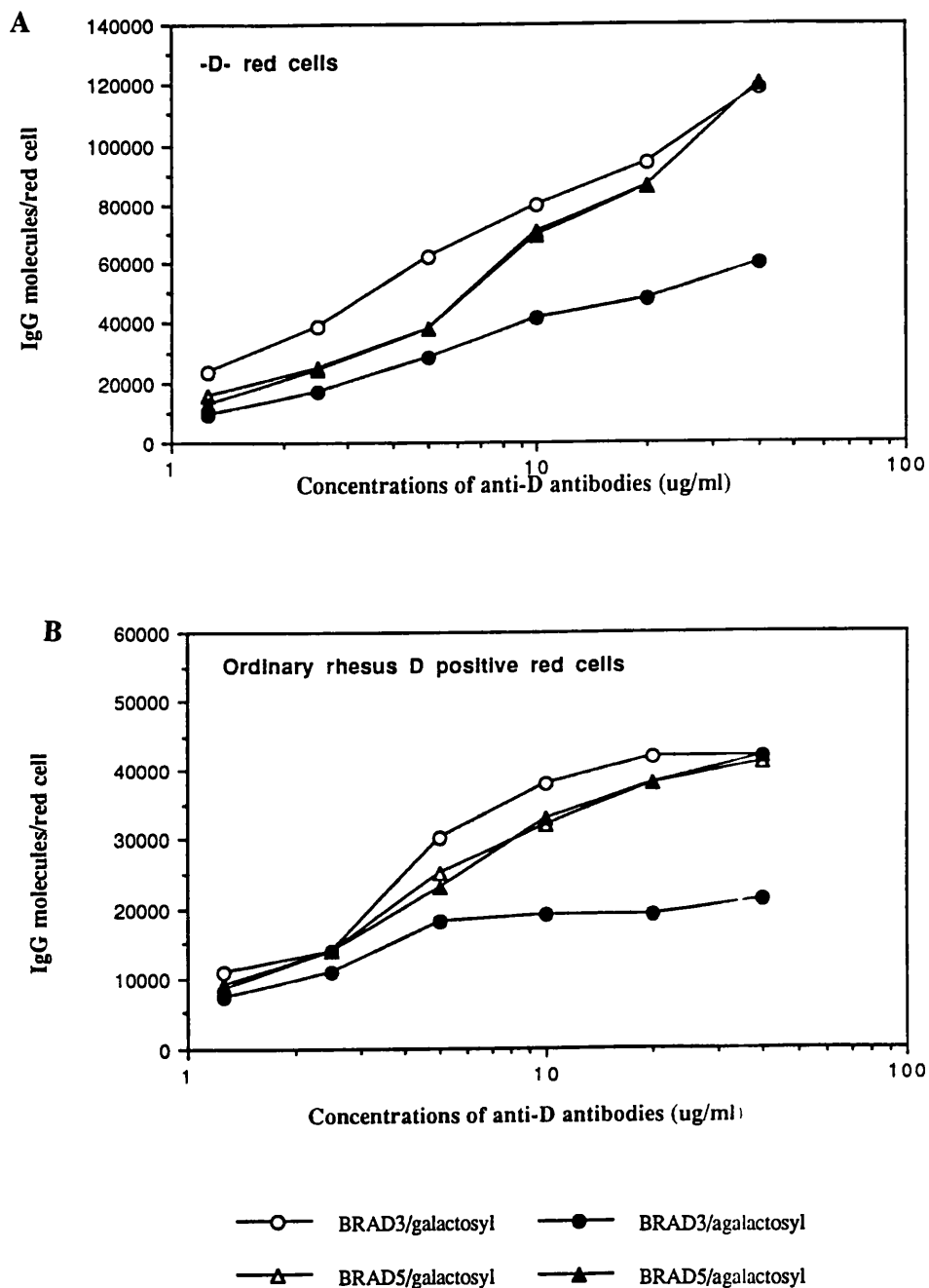
6.3. Results

6.3.1. Rosette formation

6.3.1.1. Determination of the number of red cell-bound IgG anti-D antibody molecules

Human IgG1 and IgG3 monoclonal anti-D antibodies (fully galactosylated) were used to sensitize human -D- red cells and ordinary RhD⁺ red cells. It was found that each of the ordinary RhD⁺ red cells was maximally sensitized at 40 µg/ml with about 40,000 IgG1 or IgG3 anti-D antibody molecules (Fig. 6.3A); whereas the number of IgG1 or IgG3 anti-D antibody molecules bound to each of the -D- red cells at 40 µg/ml was much higher, which was about 120,000 molecules per -D- red cell (Fig. 6.3B). This demonstrated that -D- red cells indeed have higher capacity to bind anti-D antibodies than ordinary RhD⁺ red cells. On the other hand, when -D- red cells and ordinary RhD⁺ red cells were incubated with agalactosylated human IgG1 and IgG3 monoclonal anti-D antibodies, it was found that the number of agalactosylated IgG1 anti-D antibody bound to each of the -D- red cells or ordinary RhD⁺ red cells was the same as the native galactosylated IgG1 anti-D antibody (Figs. 6.3A&B); in contrast, the number of agalactosylated IgG3 anti-D antibody molecules bound to each of the -D- red cells or ordinary RhD⁺ red cells was about half that of the native galactosylated IgG3 anti-D antibody (Figs. 6.3A&B). This indicated that either the binding capacity of IgG3 anti-D antibody to red cells had been reduced upon

Figure 6.3 Number of red cell-bound IgG anti-D antibody molecules



A. Sensitization of -D- red cells with galactosyl (open symbols) and agalactosyl (closed symbols) IgG1 (Brad 5) and IgG3 (Brad 3) anti-D antibodies. B. Sensitization of ordinary rhesus D positive red cells with galactosyl and agalactosyl IgG1 (Brad 5) and IgG3 (Brad 3) anti-D antibodies. Red cells were incubated with anti-D antibodies at various concentrations between 1.25 $\mu\text{g/ml}$ and 40 $\mu\text{g/ml}$ (horizontal axis). The number of antibody molecules bound to each red cell (vertical axis) was calculated using the formula described in the method.

degalactosylation or, more likely, that the concentration of soluble IgG3 was lower after β -galactosidase treatment. However, no change has been observed with IgG1 anti-D antibody in its antigenic binding capacity. In addition, as a control, neither isotypes of anti-D antibody bound to Rh D negative red cells.

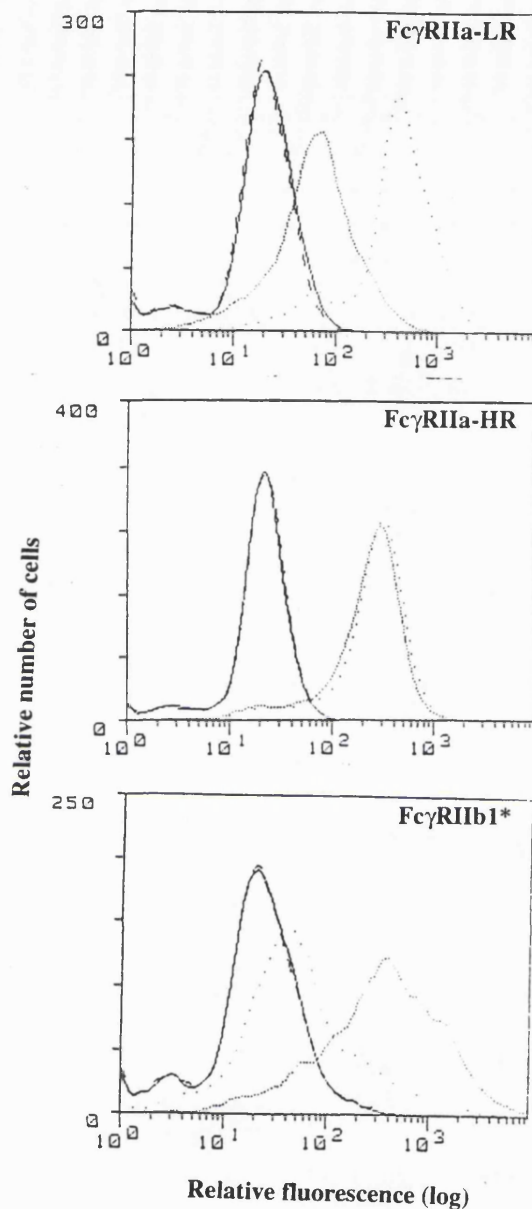
6.3.1.2. Expression of Fc γ RII on the transfectant cells

The expression levels of hFc γ RII on transfected mouse 3T6 cells were examined by indirect immunofluorescence with two specific monoclonal anti-Fc γ RII antibodies, IV.3 and 41H16. In Fig. 6.4, the relative fluorescence intensities represented the reactivities of antibodies with Fc γ RII expressed on cells. It was shown that mAb IV.3 reacted predominantly with Fc γ RIIa-LR and Fc γ RIIa-HR transfectants, and mAb 41H16 reacted mainly with Fc γ RIIa-HR and Fc γ RIIb1* transfectants. In contrast, the control antibody P3 or FITC-labelled secondary antibody did not show any binding to the transfectants. These results were consistent with the previous reports when these transfectants were established (Warmerdam *et al*, 1990 & 1993b).

6.3.1.3. Effect of IgG galactosylation change on rosette formation

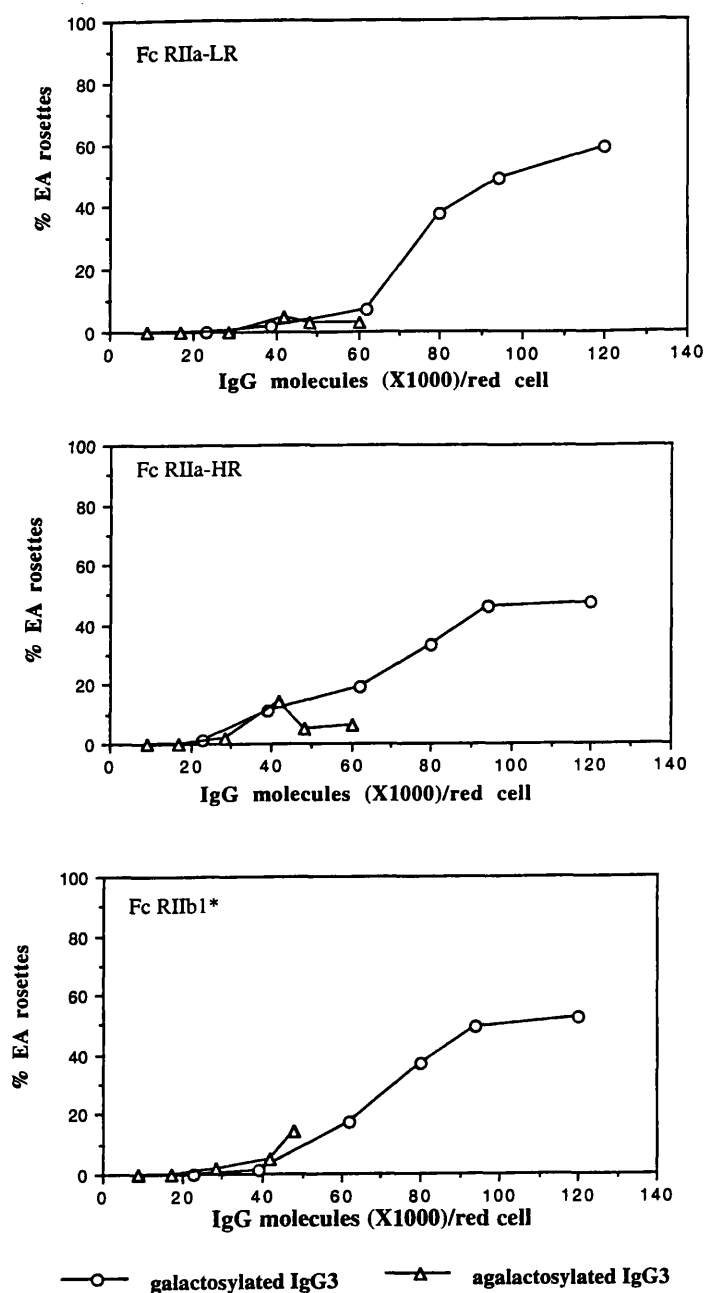
The hFc γ RII transfectants were first incubated with -D- red cells sensitized with native galactosylated IgG3 anti-D antibody. As shown in Fig. 6.5, significant rosette formation was found with the hFc γ RIIa-LR and hFc γ RIIb1* transfectants when each of the -D- red

Figure 6.4 Human Fc γ RII expression levels on three transfectants



Cells were incubated with mAb IV.3 ($\cdot \cdot \cdot \cdot$), mAb 41H16 ($\cdots \cdots$), control antibody P3 ($- - - -$) or medium alone ($—$), followed by FITC-labelled goat anti-mouse IgG antiserum. Fluorescence was recorded as arbitrary units on a logarithmic scale (horizontal axis) and plotted against the relative cell number (vertical axis). Panels represent three Fc γ RII transfectants, Fc γ RIIa-LR, Fc γ RII α -HR and Fc γ Rb1*.

Figure 6.5 Effect of IgG galactosylation changes on rosette formation through Fc γ RII with -D- RBC



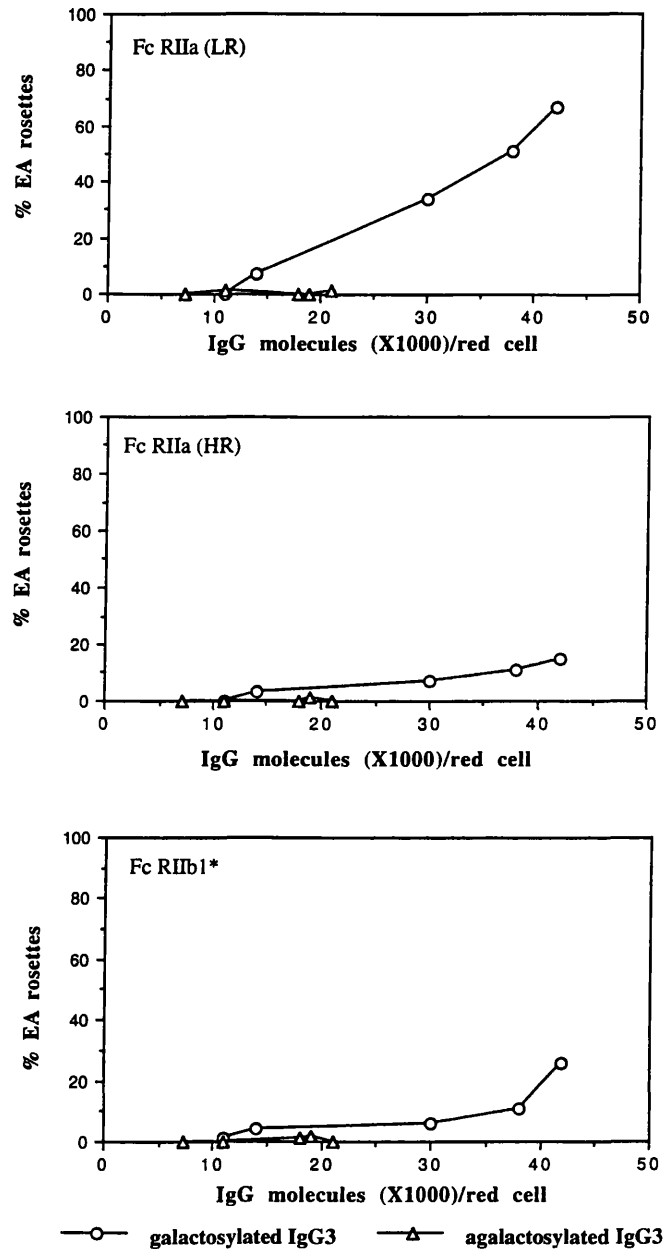
Three Fc γ RII transflectants (Fc γ RIIa-LR, Fc γ RIIa-HR and Fc γ RIIb1*) were incubated with -D- red cells sensitized with various number of galactosyl (circle symbol) and agalactosyl (triangle symbol) IgG3 molecules. The percentage rosettes (vertical axis) were counted under a microscope, and plotted against the number of IgG molecules bound per -D- red cell (horizontal axis).

cells was sensitized with over 60,000 molecules of galactosylated anti-D and with the hFc γ RIIa-HR transfectant when each of the -D- red cells was sensitized with over 40,000 molecules. At a sensitization of 120,000 molecules per red cell, about 50% of the hFc γ RII transfectants formed rosettes. In comparison, the hFc γ RII transfectants were incubated with -D- red cells sensitized with agalactosylated IgG3 anti-D antibody. As demonstrated in Fig. 6.5, at the same levels of red cell sensitization (20,000-60,000 molecules per red cell), no significant difference was noted between galactosylated and agalactosylated IgG3 anti-D antibody in their ability to form rosettes with hFc γ RII transfectants.

The rosetting assay could not be carried out using IgG1 anti-D antibody due to the shortage of the -D- red cells; the incidence of -D-/-D- individuals has been estimated at about 1 in 6 million (B. Kumpel, personal communication). Therefore, ordinary RhD⁺ red cells were used instead. No rosettes were found (data not shown) with any of hFc γ RII transfectants when incubated with ordinary red cells sensitized with either galactosylated or agalactosylated IgG1 anti-D antibody.

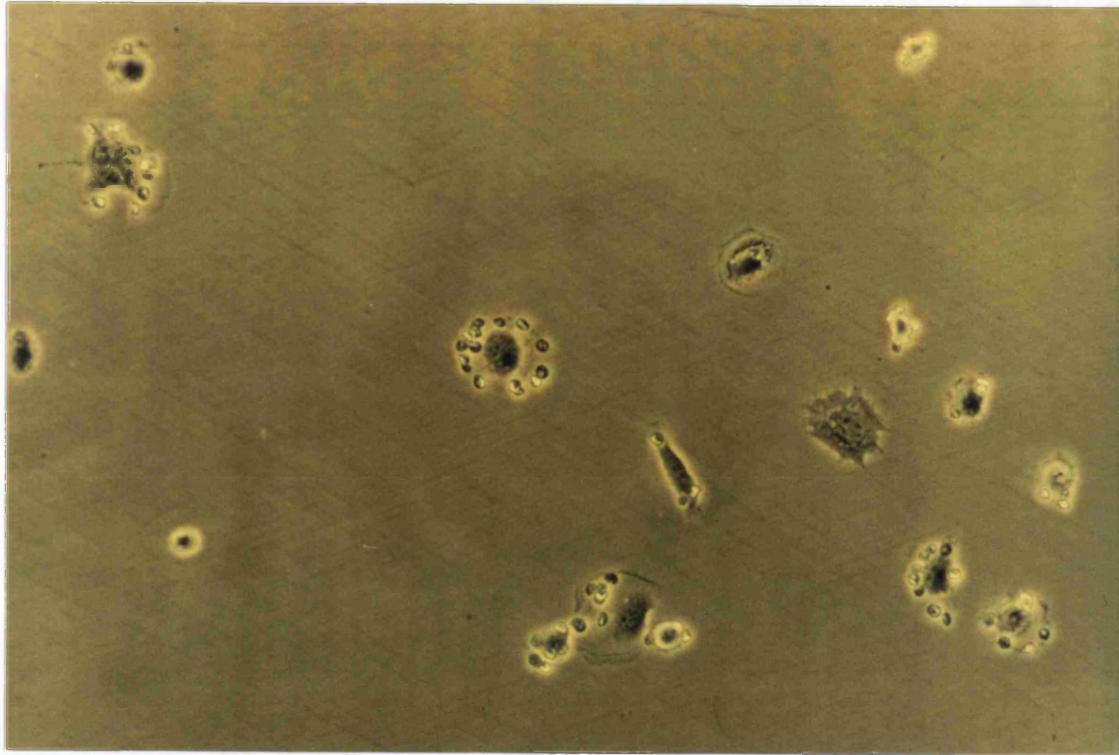
However, it was interesting to note that about 50% of hFc γ RIIa-LR, 15% of hFc γ RIIa-HR and 25% of hFc γ RIIb1* transfectants formed rosettes with ordinary RhD⁺ red cells maximally sensitized (42,000 molecules per red cell) with native galactosylated IgG3 anti-D (Fig. 6.6). The rosettes formed with hFc γ RII transfectants using ordinary RhD⁺ red cells as indicator cells are illustrated in Fig. 6.7. In contrast to galactosylated IgG3 anti-D antibody, no rosettes were found with all three types of hFc γ RII transfectants when ordinary red

Figure 6.6 Effect of IgG galactosylation changes on rosette formation through Fc γ RII with ordinary RhD⁺ RBC



Three Fc γ RII transflectants (Fc γ RIIa-LR, Fc γ RIIa-HR and Fc γ RIIb1*) were incubated with ordinary RhD positive red cells sensitized with various number of galactosyl (circle symbol) and agalactosyl (triangle symbol) IgG3 molecules. The percentage rosettes (vertical axis) were counted under a microscope, and plotted against the number of IgG molecules bound per ordinary RhD positive red cell (horizontal axis).

Figure 6.7 Rosettes formed with hFcγRII transfectants using ordinary RhD⁺ RBC as indicator cells



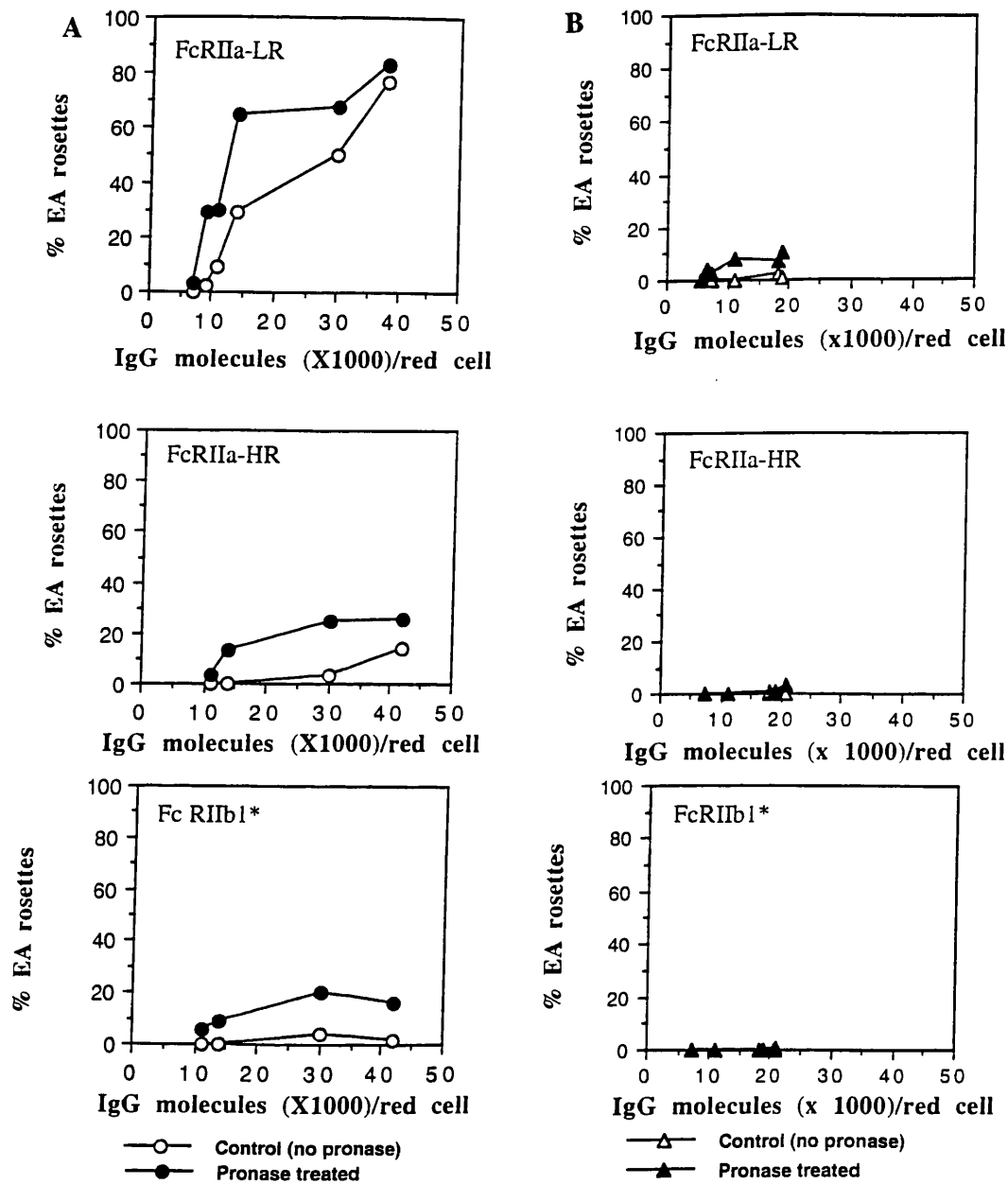
Cells were fixed with 2.5% glutaraldehyde and stained with 0.4% trypan blue. The photo was taken under a phase-contrast microscope at 40x magnification.

cells were maximally sensitized (21,000 molecules per red cell) with agalactosylated IgG3 anti-D antibody (Fig. 6.6). Therefore, at the level of red cell sensitization with 21,000 molecules, a 15% difference in number of rosettes has been observed with hFcγRIIa-LR transfectant between galactosylated and agalactosylated IgG3 anti-D antibody (Fig. 6.6). However, such a difference found with hFcγRIIa-HR and hFcγRIIb1* was minor.

6.3.1.4. Effect of pronase on rosette formation between hIgG3-sensitized erythrocytes and hFcγRII transfectants

The three hFcγRII transfectants were incubated with pronase at 50 µg/ml at 37°C for 30 minutes, and then used in rosette formation with ordinary RhD⁺ red cells sensitized with galactosylated and agalactosylated IgG3 anti-D antibodies (BRAD-3) studied. Figure 6.8A illustrates the percentage rosettes formed between the three types of hFcγRII transfectants (pronase treated and non-treated) and red cells sensitized with galactosylated IgG3 anti-D; whereas Figure 6.8B demonstrates the percentage rosettes formed between the three transfectants (pronase treated and non-treated) and red cells sensitized with agalactosylated IgG3 anti-D. It was found that treatment of all three types of hFcγRII transfectants with 50 µg/ml of pronase resulted in a comparative increase in percentage rosettes with galactosylated IgG3 anti-D antibody (Fig. 6.8A). Such an increase in percentage rosettes seems to be more obvious with the hFcγRIIa-HR and hFcγRIIb1* transfectants. However, pronase treatment of the three transfectants only caused a slight increase, when compared with non-

Figure 6.8 Effect of pronase on rosette formation



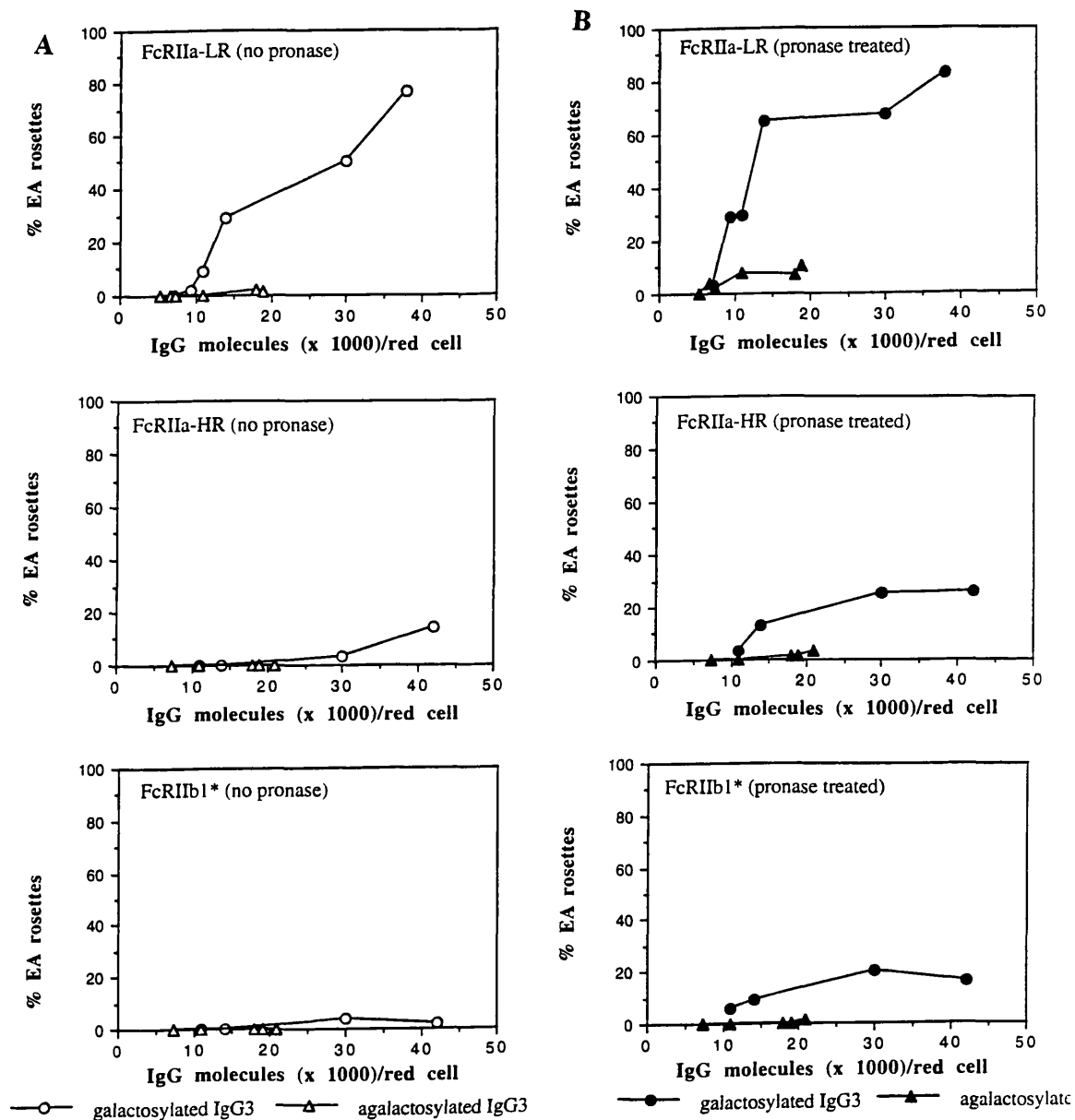
A. Ordinary RhD⁺ red cells sensitized with galactosyl (circle symbol) IgG3 anti-D mAb were incubated with three hFcγRII transflectants (hFcγRIIa-LR, hFcγRIIa-HR and hFcγRIIb1*) treated with (closed circle symbol) and without (open circle symbol) pronase. B. Ordinary RhD⁺ red cells sensitized with agalactosyl (triangle symbol) IgG3 anti-D mAb were incubated with three transflectants treated with (closed triangle symbol) and without (open triangle symbol) pronase. The percentage rosettes (vertical axis) was assessed using a microscope and plotted against the number of IgG molecules per red cell.

treated control, in percentage rosettes with red cells sensitized with agalactosylated IgG3 anti-D (Fig. 6.8B). Therefore, there was a marked reduction in the rosette formation between red cells sensitized with agalactosylated IgG3 anti-D and all three pronase-treated transfectants, using ordinary RhD⁺ red cells, at comparable levels of red cell sensitization, to that of galactosylated IgG3 anti-D (Fig. 6.9).

6.3.1.5. Effect of pronase on the expression of hFcγRII on the transfectants

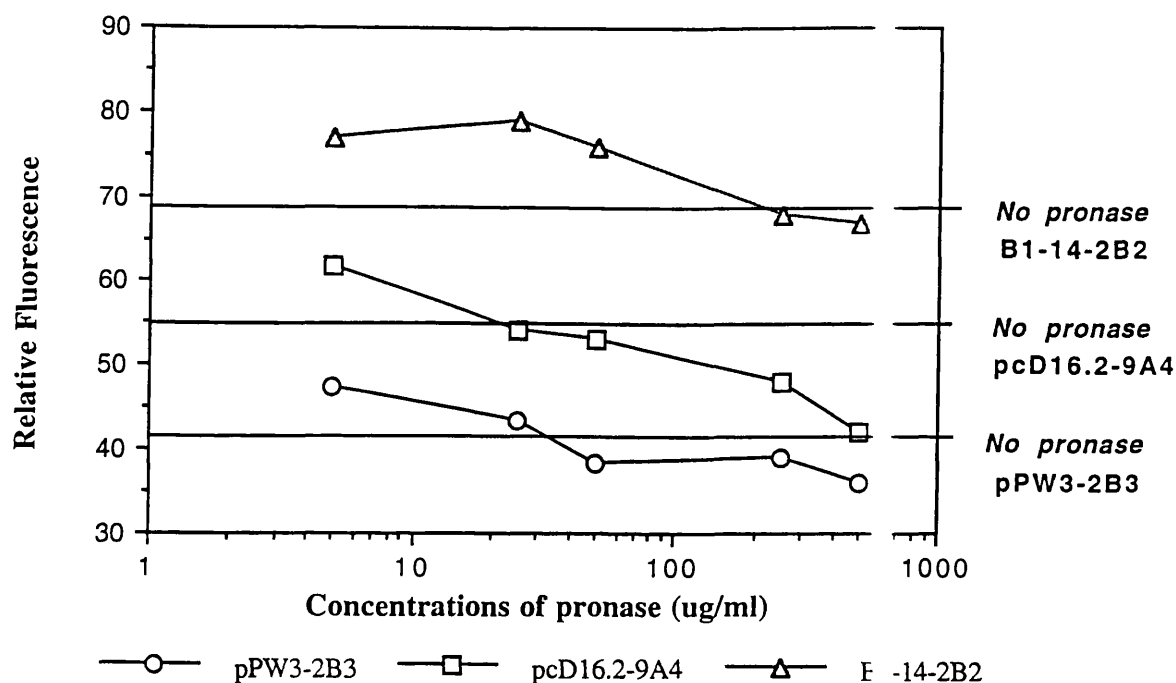
To understand how pronase enhanced the rosette formation, the expression levels of hFcγRII on the transfectants were re-examined after pronase treatment by indirect immunofluorescence using mAb IV.3 or mAb 41H16. In Figure 6.10, the relative fluorescence, which represents the reactivity of anti-hFcγRII antibody with hFcγRII expressed on the transfected cells, was plotted against the concentrations of pronase. It was found that the relative fluorescence densities of the pronase treated transfectants was slightly altered when compared with the non-pronase treated control. When the transfectants were treated with pronase at lower concentrations, i.e., 5 µg/ml or 25 µg/ml, the relative fluorescence densities were a little higher than the non-treated control. On the other hand, when the transfectants were treated with pronase at higher concentrations, i.e., 250 µg/ml or 500 µg/ml, the relative fluorescence densities tended to be lower than non-treated control. However, when the three transfectants were treated with pronase at about 50 µg/ml, the relative fluorescence densities did not differ from the non-treated control.

Figure 6.9 Rosette formation between pronase-treated hFcγRII transflectants and red cells sensitized with IgG3 anti-D mAb



A. The galactosyl IgG3 anti-D mAb (open circle symbol) was compared with agalactosyl IgG3 anti-D mAb (open triangle symbol) in their ability to form rosettes with three transflectants treated without pronase. B. The galactosyl IgG3 anti-D mAb (closed circle symbol) was compared with agalactosyl IgG3 anti-D mAb (closed triangle symbol) in their ability to form rosettes with three transflectants treated with pronase. Ordinary RhD⁺ red cells were used as indicator cells.

Figure 6.10 Effect of pronase on the expression of hFcγRII on transfectant cells



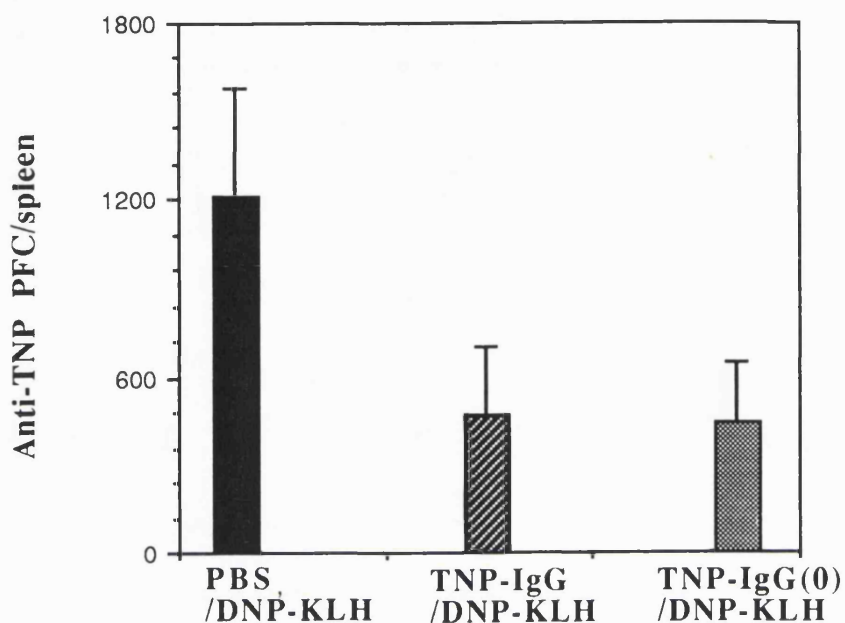
Three hFcγRII transfectants, hFcγRIIa-LR (circle symbol), hFcγRIIa-HR (square symbol) and hFcγRIIb1* (triangle symbol), were incubated with pronase at various concentrations ranging from 5 μg/ml to 500 μg/ml. The pronase-treated and non-treated transfectants were then incubated with either mAb IV.3 or mAb 41H16, followed by FITC-labelled goat anti-mouse IgG antiserum. The horizontal lines represent the relative fluorescence intensities of the three transfectants treated without pronase.

This concentration (50 µg/ml) of pronase has also been reported in a previous study to have no effect on the expression of hFcγRII on cell lines (Tuijnman *et al* ,1990).

6.3.2. Effect of IgG galactosylation change on the *in vivo* induction of hapten specific tolerance by haptenated isologous IgG

In this experiment, three groups of mice were primed with an intravenous injection of either PBS only, or 0.2 mg of TNP₅-IgG conjugate which was treated with and without β-galactosidase. Subsequently, all mice were challenged with an intraperitoneal injection of 0.2 mg of DNP-KLH emulsified in CFA. The anti-TNP response in spleen was measured 5 days later using plaque forming assay. This experiment was repeated twice, and a similar pattern of result was obtained though the overall values of the two individual experiment was quite different. Data from the two experiments were therefore pooled together, and expressed as the geometric mean and the standard error (s.e.) in each group (Fig. 6.11). It was shown that the number of anti-TNP plaque forming cells in mice primed with galactosylated TNP₅-IgG conjugates was half that seen in PBS-primed control mice. The difference between these two groups was found to be statistically significant ($p < 0.05$, the Mann-Whitney test). When mice were primed with agalactosylated TNP₅-IgG conjugates, a similar degree of depression in number of anti-TNP plaque forming cells was observed. No significant difference has been found in the number of PFC between the group of mice primed with galactosylated TNP₅-IgG and that of agalactosylated TNP₅-IgG (the Mann-Whitney

Figure 6.11 Effect of IgG galactosylation change on the induction of a hapten specific tolerance *in vivo*.



Three groups of C57 black mice (n=4/group) were injected intravenously with either PBS alone (left hand bar), or 0.2 mg of galactosyl (middle bar) and agalactosyl (right hand bar) TNP₅-IgG conjugates. All mice were then received an intraperitoneal injection of 0.2 mg of DNP-KLH emulsified in CFA. The number of anti-TNP plaque forming cells in the spleens were measured 5 days later. The experiment was repeated twice, and the results were expressed as the geometric mean of the pooled data plus the standard error.

test). Therefore, the galactose removal from pregnant murine IgG by galactosidase treatment did not affect its ability to induce tolerance to hapten *in vivo*.

6.4. Discussion

The role of agalactosyl IgG remained obscure almost ten years after its discovery. One of the reasons was that agalactosyl IgG cannot be easily purified. Tsuchiya *et al* (1989) had discovered a β -galactosidase enzyme from *Streptococcus* which could remove all the galactose residues from intact human IgG. Using this approach, they demonstrated that agalactosylated IgG bound less effectively to the Fc γ RI expressed on U937 cells than galactosylated IgG. In the present study, the same β -galactosidase enzyme was used to remove galactose from the galactosylated IgG monoclonal anti-D antibodies and murine IgG of pregnant mice. Surprisingly, such a treatment did not lead to a complete removal of galactose residues from IgG molecules as determined by G(0) plate assay. This may be due to the absence of sialidase in the digest, which had been used in combination with galactosidase in the previous study by Tsuchiya *et al* (1989). However, in preliminary experiments, sialidase had caused a severe precipitation of IgG after use, and it would result in exposure of terminal galactose on all oligosaccharides on IgG, whereas naturally occurring agalactosyl oligosaccharides are seen almost exclusively on the CH2 domain where there is little capping with sialic acid. Therefore sialidase was omitted in subsequent experiments.

In the first part of the study, we compared the galactosylated

and partly agalactosylated IgG monoclonal anti-D antibodies in their ability to bind to human Fc γ RII on the transfectants by rosette formation. Since hFc γ RII is a low affinity receptor, which has been found only to form stable rosettes at high levels of red cell sensitization (Dr van de Winkel, personal communication), we used -D- red cells to achieve high levels of red cell sensitization with monoclonal anti-D antibodies (Dr Kumpel, personal communication). However, the amount of -D- red cells we obtained was only enough to study one of the anti-D monoclonal antibodies, which was chosen to be IgG3 monoclonal anti-D, because it had previously been shown to have a greater capacity than IgG1 monoclonal anti-D to interact between red cells and human monocytes or cell lines (U937 and Daudi) expressing human Fc γ R (Kumpel and Hadley, 1990b). Surprisingly, the results were complicated by the fact that agalactosylated IgG3 anti-D antibody showed reduced ability to sensitize red cells. When galactosylated IgG3 anti-D was compared with its agalactosylated form at similar levels of red cell sensitization, below 60,000 IgG molecules per red cell, few rosettes were observed with the three hFc γ RII transfectants and with either preparation of anti-D. Since the maximal level of red cell sensitization can be achieved with agalactosylated IgG3 anti-D was about 60,000 molecules, we were unable to predict the rosette formation with the hFc γ RII transfectants at high levels of red cell sensitization such as above 60,000 IgG molecules per red cell. Therefore, we aim to study IgG1 anti-D antibody when -D- red cells are available, because agalactosylated IgG1 anti-D antibody was found to bind as well to red cells as its original galactosylated form, and high levels of red cell

sensitization i.e. above 60,000 molecules per red cell can be achieved. In an attempt to circumvent these problems, we sensitized ordinary RhD⁺ red cells with IgG1 anti-D antibody of both forms. However, no rosette formation with hFcγRII transfectants was found at any level of red cell sensitization. Interestingly, at 20,000 molecules of galactosylated IgG3 anti-D per red cell, ordinary RhD⁺ red cells formed 15% of rosettes with the hFcγRIIa-LR transfectant, but at the same level of red cell sensitization, agalactosylated IgG3 anti-D did not form any rosettes with hFcγRIIa-LR transfectant. With hFcγRIIa-HR and hFcγRIIb1* transfectants, also at 20,000 IgG molecules per red cell, the agalactosylated IgG3 anti-D showed little difference from its galactosylated form in rosette formation. But when the affinity of hFcγRII had been increased by pronase treatment, the difference in rosette formation between agalactosylated and galactosylated IgG3 became more marked with all three transfectants. Although it was observed that at comparable levels of red cell sensitization, i.e. 20,000 molecules of galactosylated IgG3 anti-D per red cell, -D- red cells did not form any rosettes with the hFcγRIIa-LR transfectant whereas ordinary RhD⁺ red cells did, the explanation for these apparently conflicting results may have been because a certain proportion of -D- red cells had haemolysed during the experiment (they have been frozen, thawed, and sent by mail) so that the number of rosettes formed was underestimated (Dr Kumpel, personal communication), whereas the ordinary RhD⁺ red cells were very fresh. Interpretation of the results of experiments using -D- red cells should therefore be made with caution. Therefore, it is concluded that there is likely a difference between agalactosylated IgG3 anti-D antibody and its

galactosylated form in their ability to interact with hFcγRII expressed on the transfectants. Nevertheless, more experiments need to be carried out to further confirm the results.

It is intriguing that the agalactosylated IgG3 anti-D antibody was found to bind less well to the red cells than its native galactosylated form. This disturbance in antigenic binding ability of agalactosylated IgG3 could not be explained by the degradation of IgG caused by contaminating proteases in the β-galactosidase enzyme preparation, since the antigenic binding ability of agalactosylated IgG1 anti-D antibody was not altered. It is possible that the longer hinge region of human IgG3 molecule may be of certain importance in stabilizing the conformation of the antigen-binding sites in the Fab region. Thus, depletion of galactose residues from the carbohydrate chains in the CH2 domain of IgG may affect the configuration of both the CH2 domain and the antigen-binding sites of IgG3 molecule, but affect IgG1 molecule only in the CH2 domain.

It has previously been reported that treatment of monocytes or K562 cells with proteolytic enzymes, such as pronase or trypsin, could enhance both the affinity of FcγRII for human IgG complexes and the signalling via this receptor (van de Winkel *et al*, 1989; Debets *et al*, 1990). A subsequent study reported that treatment with proteolytic enzymes, such as 50 µg/ml of pronase, only affected the affinity of FcγRII on myeloid cells but not on B cells (Tuijnman *et al*, 1990). In the present study, it was found that treatment of hFcγRII transfectant cells with 50 µg/ml of pronase enhanced rosette formation considerably with ordinary RhD⁺ red cells sensitized with galactosylated IgG3 anti-D antibodies, but to a much lesser extend

with agalactosylated IgG3 anti-D antibodies. Since no apparent change in expression of hFcγRII was observed with 50 μg/ml of pronase treatment, such an elevation in rosette formation may be due to increased affinity of hFcγRII. Interestingly, such a pronase treatment increased not only the affinity of hFcγRIIa-LR, hFcγRIIa-HR, but also hFcγRIIb1* which is generally present on B cells, in contrast to the finding by Tuijnman *et al* (1990). It might be that the hFcγRII expressed on the transfectant cells is somewhat different from the FcγRII present on the B cell lines examined in the previous study. It is not clear how pronase increased the affinity of hFcγRII. A previous study had shown that enzymes, such as neuraminidase or bromelain, could enhance rosette formation by reducing the negative charge of the effector cells (Kumpel and Hadley, 1990b).

In the second part of the study, we used murine pregnancy IgG as a source of fully galactosylated IgG, and compared galactosylated and agalactosylated TNP-IgG in their ability to tolerize mice to TNP before challenge with DNP-KLH, using anti-TNP plaque forming assay. In these experiments, we did not detect any loss of tolerizing ability attributable to removal of galactose from IgG. However, this may be due to ineffective galactose removal from IgG in the agalactosyl IgG preparation, and therefore the ability of IgG to bind FcγR on B cells and subsequently to confer a negative signal has not been apparently affected. Nevertheless, a more recent study performed by Rademacher *et al* (1994), using the same agalactosyl IgG preparation procedure, showed that agalactosyl IgG autoantibodies are more effective than the galactosylated form in precipitating murine collagen-induced arthritis. Moreover, this effect of agalactosyl IgG

has been further demonstrated to be IgG subclass-dependent (Dr Rademacher, personal communication). Thus removal of galactose from monoclonal antibodies to conformational epitopes on type II collagen, caused IgG2a and IgG2b antibodies to develop increased arthritogenicity and to drive antibody production in the recipient, while removal of galactose from an IgG1 monoclonal antibody increased its negative regulatory function. In this respect, the effective tolerizing ability of agalactosylated TNP-IgG *in vivo* might be an effect of murine IgG1 subclass. Therefore, it would be interesting to further carry out the *in vivo* tolerization experiments with purified murine IgG subclasses, and also to repeat the *in vitro* FcγR binding studies with murine FcγRII-transfected cell lines and with murine IgG subclasses.

Chapter 7.

General Discussion

7.1. The disease associations of agalactosyl IgG

Agalactosyl IgG is defined as IgG molecules containing no terminal galactose on the bi-antennary *N*-linked oligosaccharides at asparagine 297 in the C γ 2 domain on Fc region. It is now well established that the levels of agalactosyl IgG are raised in a specific group of diseases including rheumatoid arthritis, tuberculosis, Crohn's disease, and leprosy during erythema nodosum leprosum (ENL). In this study, two more diseases were found also having raised levels of agalactosyl IgG, that is, sarcoidosis and Takayasu's arteritis.

It was previously shown that the levels of agalactosyl IgG correlated with disease activity in both adult and juvenile onset rheumatoid arthritis (Parekh *et al*, 1988b), and during pregnancy of arthritic women (Rook *et al*, 1991c). The decrease in agalactosyl IgG during gestation correlated with the pregnancy-induced remission of the disease, and the sharp increase in agalactosyl IgG post-partum correlated with the relapse of the disease. This is also true during pregnancy of mice with pristane-induced arthritis (Thompson *et al*, 1992). In this study, raised agalactosyl IgG was detected only in patients with active Takayasu's arteritis, but not in those with inactive disease. Moreover, a significant decrease in agalactosyl IgG was observed upon resolution of sarcoidosis, while a constant high level of

agalactosyl IgG was associated with the persistence of the disease.

Besides the association with disease activity, agalactosyl IgG has been found to have predictive clinical value. Raised agalactosyl IgG has been demonstrated prior to clinical onset of rheumatoid arthritis, and therefore can be considered a risk factor for the development of the disease (Schrohenloher *et al*, 1991). Agalactosyl IgG can also be used as an aid to differential diagnosis in early synovitis (Young *et al*, 1991). It was shown that most patients with early synovitis would develop rheumatoid arthritis if they had raised agalactosyl IgG at presentation. Moreover, a recent publication from this study has shown that rheumatoid arthritis patients with elevated levels of agalactosyl IgG in the early stage of the disease could have a more poor prognosis six years later than those with normal levels of agalactosyl IgG (van Zeben *et al*, 1994). To fulfill these clinical significance of agalactosyl IgG, an efficient routine test is important. The method developed in this study, the G(0) plate assay, is therefore of particular value for the rapid detection of agalactosyl IgG in patients sera.

7.2. The cause of the increase in agalactosyl IgG

Although the actual cause for the increase in agalactosyl IgG in disease states remains unknown, an environmental factor is strongly suspected. A rise in percentage agalactosyl IgG amongst family members who are not blood relatives has been noted in rheumatoid arthritis (Sumar *et al*, 1991), spouses of members of families with a high incidence of autoimmune disease (Tomana *et al*, 1992), and

spouses of patients with sarcoidosis (Fidler *et al*, submitted). This environmental factor seems to be transmissible in a way, however, remains to be identified.

The diseases where raised agalactosyl IgG was found are, either known to be caused by mycobacteria or suspected to have a mycobacterial aetiology. The levels of agalactosyl IgG were shown to be normal in several human viral infections (Rademacher *et al*, 1988). Thus it was suspected that raised agalactosyl IgG may be a marker of an infection with mycobacteria (Rademacher *et al*, 1988), which possibly are forms defective in cell wall, extremely slow-growing and undetectable by conventional methods such as staining or culture (Rook and Stanford, 1992). These diseases with raised agalactosyl IgG have been further associated by having several other common features, such as arthritis and autoantibody production. It was argued that autoimmunity is secondary to slow bacterial infection, and the increase in agalactosyl IgG is resulted from the chronic release of cytokines, particularly IL-6, during T-cell-mediated inflammation in the primary site of infection, such as lungs or gut (Rook and Stanford, 1992).

However, raised agalactosyl IgG does not seem to be only associated with a mycobacterial infection, which has also been recently demonstrated in goats and sheeps infected with potentially arthritogenic viruses, such as CAEV and maedi-visna (McCulloch *et al*, in press). It was argued that raised agalactosyl IgG is associated potentially arthritogenic bacterial or viral infections, and is not necessarily an indicator of mycobacterial aetiology. However, this suggestion remains to be tested in human conditions.

7.3 The regulation of agalactosyl IgG

The precise mechanism that regulates the production of agalactosyl IgG is unclear. However, the increase in agalactosyl IgG is likely to result from the reduced galactosyltransferase activity in antibody-producing B cells (Axford *et al*, 1987), rather than the extracellular degradation by β -galactosidase during circulation, since lymphocytes isolated from the peripheral blood of patients with rheumatoid arthritis secreted agalactosyl IgG (Bodman *et al*, 1992). It has been reported that the β -galactosyltransferase in B cells specifically transfers UDP-galactose onto an asialo-agalactosyl IgG (Furukawa *et al*, 1990). The affinity of β -galactosyltransferase for UDP-galactose in the B lymphocytes of patients with rheumatoid arthritis is lower than in B lymphocytes from a control group. Further, the specific transfer activity of this galactosyltransferase from B lymphocytes of RA patients towards asialo-agalactosyl IgG is found to be reduced to 50-60% of controls (Furukawa *et al*, 1990). More recently, the relationship between galactosyltransferase activity and the levels of agalactosyl IgG has been found to be negative and linear in the patients with rheumatoid arthritis, but positive and linear in the control population (Axford *et al*, 1992). Although the increase in agalactosyl IgG in rheumatoid arthritis is correlated with the decreased activity of galactosyltransferase in B cells, it is not known whether the same is true in other diseases with raised agalactosyl IgG.

Since IL-6 is an important B cell differentiating factor, and has been found capable of altering the activity glycosyltransferases,

including galactosyltransferase, it is possible that IL-6 plays a role in the regulation of agalactosyl IgG (Rook *et al*, 1992a). Evidence in support this suggestion are as follows. Strikingly raised agalactosyl IgG has been found in transgenic mice over-expressing human IL-6 gene (Rook *et al*, 1991a). Direct injection of recombinant IL-6 into normal DBA/1 mice resulted in an increase in serum agalactosyl IgG (Hitsumoto *et al*, 1992). Levels of IL-6 in the peritoneal cavity correlated with the serum levels of agalactosyl IgG in mice received an intraperitoneal injection of pristane (Hitsumoto *et al*, 1992). The levels of agalactosyl IgG significantly correlated with the levels of IL-6 in sera from patients with sarcoidosis. A simultaneous rise in agalactosyl IgG and IL-6 was found in sera from patients with Castleman's disease (Nakao *et al*, 1991).

However, IL-6 is unlikely to be the only regulator of agalactosyl IgG production, identification of other factors would be interesting. The dynamic change in serum levels of agalactosyl IgG during pregnancy may suggest the involvement of endocrinological factors (Rook *et al*, 1992a). The level of agalactosyl IgG falls to very low levels during pregnancy in mice and human (Rook *et al*, 1991c; Thompson *et al*, 1992), and rises again rapidly post-partum. The same pattern of variation in levels of agalactosyl IgG was also seen in pregnant patients with rheumatoid arthritis (Rook *et al*, 1991c), except that all the agalactosyl IgG values were higher. Therefore, it seems to suggest that the mechanisms which lower the levels of agalactosyl IgG in pregnancy are distinct from the mechanisms which set the levels too high in rheumatoid arthritis. The raised agalactosyl IgG in mice with collagen-induced arthritis (Rook *et al*, 1991c), or pristane-

induced arthritis (Thompson *et al*, 1992) also falls in pregnancy. Moreover, the same was seen in pregnant patients with Crohn's disease (Pilkington *et al*, submitted).

It is not known what causes the variation of agalactosyl IgG in normal individuals. It may be explained by the age-related expression of the galactosyltransferase activity in B cells, but such a correlation has not yet been observed (Furukawa *et al*, 1990). It would be interesting to look at the variation IL-6 levels and endocrinological factors at different ages in normal individuals in association with the levels of agalactosyl IgG.

7.4. The biological properties of agalactosyl IgG

It is not known whether raised agalactosyl IgG is a by-product of the disease process or involved in the pathogenesis of the diseases. The rise in agalactosyl IgG prior to the onset of rheumatoid arthritis (Schrohenloher *et al*, 1991), and the circumstantial correlation between the levels of agalactosyl IgG and the disease activity seems to suggest that agalactosyl IgG is directly linked with the disease. Recently, it has been shown that agalactosyl IgG autoantibodies are directly associated with pathogenicity in murine collagen-induced arthritis (Rademacher *et al*, 1994). Thus, the occurrence of an acute synovitis in T-cell-primed (by intradermal injection of heat-denatured type II collagen) mice was enhanced by subsequent administration of IgG containing agalactosyl anti-collagen autoantibodies. Therefore, the nonpathogenic doses of autoantibodies were made pathogenic by removing their galactose contents. However, it is not clear what are

the mechanisms.

In this study, it was shown that removal of galactose from IgG anti-D antibodies could result in their reduced ability to form rosettes with human Fc γ RII transfectants, especially with the Fc γ RIIa-LR subclass transfectant. Since Fc γ RII are the receptors expressed on B cells, the reduced binding of agalactosyl IgG to these receptors may therefore release the negative signal normally exerted by cross-linking the surface Ig and Fc receptors on B cells, and convert it into a positive signal. However, when agalactosyl IgG was tested for its ability to induce hapten specific tolerance in mice, no difference has been observed between agalactosyl IgG and its more galactosylated form. In addition to Fc γ RII, we also tested agalactosyl IgG in their ability to interact with the other two classes of Fc receptors, Fc γ RI and Fc γ RIII (Wang *et al*, in preparation). It was found that the rosette formation and phagocytosis mediated by Fc γ RI and agalactosyl IgG was reduced in comparison with that by galactosyl IgG. Further, ADCC mediated by Fc γ RIII and agalactosyl IgG was also reduced. These results are consistent with the previous observation by Tushiya *et al* (1989), in which agalactosyl IgG was found to bind less effectively with the Fc γ RI on a monocytic cell line. These results imply that agalactosyl IgG may have an effector function as well as a regulatory function. However, further study on the Fc γ RII is needed since conflicting results was obtained from the *in vitro* and *in vivo* system.

In addition, it has also been suggested that the exposure of terminal GlcNAc resulting from the lack of galactose allows this sugar to interact with lectin-like membrane receptors or soluble mediators

(such as mannose-binding protein), which alter the regulatory or functional role of the antibody (Rademacher, 1991).

Agalactosyl IgG was defined ten years ago, but after an initial phase of excitement, many workers lost interest in it. This could be an error of judgement, and the story is just beginning. Agalactosyl IgG is proving to be an important marker of a particular type of pathological process. Perhaps it is acting as a measure of a pattern of chronic cytokine (Rook *et al*, 1991a) and endocrine (Rook *et al*, 1991c) activity, equivalent to measuring the area under a complex curve. Considerable data from a variety of sources now imply that agalactosyl IgG is sometimes associated with cryptic infection, and can act as a marker of populations of goats (McCulloch *et al*, in press) and humans (Tomana *et al*, 1992 & 1994) at risk of autoimmune disease, particularly arthritis. This should inspire renewed efforts to define novel infectious agents. Agalactosyl IgG may directly contribute to disease persistence and pathogenesis (Rademacher *et al*, 1994). However, the role of agalactosyl IgG in the pathogenesis of tuberculosis is not yet studied. It would be interesting to know the specificity of agalactosyl IgG, which may help us to understand its involvement in the disease process.

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APPENDICES

0.28 M cacodylate buffer, pH 6.9

Sodium Cacodylate	28.5 g
Distilled H ₂ O	250 ml
Adjust pH to 6.9 with 1N NaOH or HCl, then make up the volume to 500 ml with distilled H ₂ O.	

Complete Freund's adjuvant (CFA)

Arlacel A (Mannide Monooleate)	1.5 ml
Bayol F (paraffin oil)	8.5 ml
Mycobacterium tuberculosis (Killed and dried extract)	5 mg

0.05 M carbonate buffer (coating buffer), pH 9.6

Na ₂ CO ₃	1.59g
NaHCO ₃	2.93g
distilled H ₂ O to 1 litre (L)	

PBS

NaCl	8 g
KH ₂ PO ₄	0.2 g
Na ₂ HPO ₄	2.8 g
KCl	0.2 g
distilled H ₂ O to 1 litre (L)	

PBS/Tween (0.05%)

PBS	1 L
Tween 20	500 µl

PBS/0.05% Tween 20/1% BSA(PBS/Tween/BSA)

PBS/Tween	1 L
BSA	10g

Glycine buffer

Glycine	0.75g
NaCl	0.935g
dH ₂ O to 100 ml	
Adjust pH to 7.0 with 1N NaOH.	

0.5% Glutaraldehyde solution

25% glutaraldehyde	200μl
PBS	9.8ml

0.1M Lysine solution

Lysine	1.82g
PBS	100ml

Citrate phosphate buffer (CPB, 0.1M, pH4.1)

Solution A: Citric acid 21.02g/L

Solution B: Na₂HPO₄·2H₂O 17.79g/L

Add equal volume of solution A to solution B to obtain pH4.1.

ABTS substrate solution

ABTS	12.5mg
CPB (0.1M, pH4.1)	25ml
hydrogen peroxide	8.75μl

Stopping solution

NaF	96mg
dH ₂ O	50ml

30% Acrylamide solution

Acrylamide	29.2 g
bis-acrylamide	0.8 g
dH ₂ O to 100 ml	

Electrophoresis buffer

Glycine	14.4 g
Tris-base	3 g
SDS	1 g
dH ₂ O to 1 L	

Blot buffer

Glycine	14.4 g
Tris-base	3 g
Methanol or 740p	200 ml
dH ₂ O to 1 L	

2 x Sample buffer

Glycerol	20%
β-mercaptoethanol	10%
SDS	4.6%
Tris·HCl (pH6.8)	0.125M
Bromophenol blue	0.1%

Coomassie staining solution

Coomassie Brilliant	
Blue R 250	0.25 g
Methanol	125 ml
Glacial acetic acid	
dH ₂ O to 1 L	

Destaining solution

Methanol	100 ml
Glacial acetic acid	100 ml
dH ₂ O to 1 L	

Ponceau S stain

Ponceau S	0.2 g
3% TCA	100 ml

Trypsin/EDTA solution (sterile)

Trypsin	20mg
EDTA	20mg
dH ₂ O	100ml