Structure and expression of the β1,4-galactosyltransferase gene in relation to IgG glycosylation

by

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**ABSTRACT**

Rheumatoid arthritis (RA) is associated with an increase in the level of serum IgG glycoforms lacking terminal galactose residues (i.e., agalactosyl IgG). The agalactosyl IgG shows altered effector functions and there is evidence that it may be pathogenic. Furthermore, levels of agalactosyl IgG have been shown to have a predictive value in RA. There is evidence that the decreased galactosylation of IgG occurs as a pre-secretory event and there are several reports relating this defect to aberrant control of the enzyme β1,4-galactosyltransferase (β1,4-GalTase). This project aimed to examine the structure and expression of the β1,4-GalTase gene in human RA and also in a murine model of arthritis, MRL/Mp-lpr/lpr (MRL lpr/lpr), which shows the same defect in IgG galactosylation.

No gross structural alteration of the gene was observed in human RA nor in the MRL lpr/lpr mice, using restriction fragment length polymorphism analysis. An RNase protection assay established that there are similar levels of β1,4-GalTase gene expression in CD19⁺ cells isolated from peripheral blood of RA patients and normal healthy individuals. IgG-expressing lymphocytes isolated from spleens and lymph nodes of MRL lpr/lpr and CBA/Ca (which exhibit normally galactosylated IgG) mice also showed comparable levels of β1,4-GalTase mRNA.

The known pregnancy associated increase in IgG galactosylation was examined in the Balb/c mice. Although the β1,4-GalTase transcription was highly upregulated in the mammary gland in the third trimester of pregnancy and into lactation, no changes in the mRNA and enzyme levels were observed in the lymphocytes isolated from spleens of these mice.

The cytokines IL-6 and TNF-α are proposed as glycosylation regulating factors. In addition, IL-6 has been shown to be associated with increased agalactosyl IgG. Therefore, the level of β1,4-GalTase gene expression was
measured in IL-6 and TNF-α transgenic mice in relation to the IgG galactosylation level. In these studies, comparable levels of β1,4-GalTase mRNA were observed in the transgenics and their littermates in both cases.

Peripheral blood lymphocytes stimulated \textit{in vitro} with the mitogens PHA, phorbol ester and pokeweed, with the cytokines IL-6 and TNF-α, with the calcium ionophore ionomycin and with the cAMP-inducer forskolin, did not show altered levels of β1,4-GalTase mRNA. However, the addition of prolactin to peripheral blood B cells cultured in the presence of anti-IgM plus IL-2 resulted in a small increase in mRNA levels but with no concomitant increase in IgG galactose.

In conclusion, these studies indicate that IgG galactosylation is not regulated at the level of β1,4-GalTase gene expression.
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ABBREVIATIONS

AIDS Acquired immunodeficiency syndrome
Ala Alanine
Allo A Alloymrina dichotoma agglutinin
Asn Asparagine
Asp Aspartate
ATG Adenine thymine guanine
ATP Adenosine triphosphate
BAP Bacterial alkaline phosphatase
BIC Carbonate/bicarbonate buffer
bp Base pair
BSA Bovine serum albumin
BSII Bandeiraea simplicifolia lectin
C Complement
CD Cluster of differentiation
CH Constant region of immunoglobulin heavy chain
Ci Curie
CIA Collagen induced arthritis
CIP Calf intestinal alkaline phosphatase
CL Constant region of immunoglobulin light chain
cAMP Cyclic AMP (adenosine 3', 5'-cyclic monophosphate)
CRE cAMP response element
CREB CRE binding protein
CTP Cytidine triphosphate
Cys Cysteine
ddH₂O double-distilled water
DEPC Diethylpyrocarbonate
DMSO Dimethylsulphoxide
DN CD4 CD8 double negative
DNA Deoxyribonucleic acid
ss/ds-DNA Single stranded/double stranded-DNA
DTT Dithiothreitol
EBV Epstein Barr virus
EDTA Ethylenediaminetetraacetic acid
Eμ The human immunoglobulin heavy chain enhancer
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorobant assay</td>
</tr>
<tr>
<td>ELLA</td>
<td>Enzyme linked lectin assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>Fab</td>
<td>Antibody binding fragment</td>
</tr>
<tr>
<td>F(ab)_2</td>
<td>Divalent antigen binding fragment</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
</tr>
<tr>
<td>Fc</td>
<td>Crystallisable fragment</td>
</tr>
<tr>
<td>FcR</td>
<td>Receptor for the Fc region of immunoglobulin</td>
</tr>
<tr>
<td>FCA</td>
<td>Freund's complete adjuvant</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Fuc</td>
<td>Fucose</td>
</tr>
<tr>
<td>Gal</td>
<td>Galactose</td>
</tr>
<tr>
<td>β1,3-GalTase</td>
<td>β1,3-Galactosyltransferase</td>
</tr>
<tr>
<td>β1,4-GalTase</td>
<td>β1,4-Galactosyltransferase</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GC</td>
<td>Guanine cytosine</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte/monocyte colony stimulating factor</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's Balanced Salt Solution</td>
</tr>
<tr>
<td>HEMPAS</td>
<td>Hereditary erythroblastic multinuclearity associated with the positive acidified serum lysis test</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-2R</td>
<td>Interleukin-2 receptor</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropylthio-β-D-galactoside</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobase</td>
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<td>KDa</td>
<td>Kilodalton</td>
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<td>K_m</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>LB medium</td>
<td>Lenox broth medium</td>
</tr>
<tr>
<td>Le^a</td>
<td>Lewis antigen: Galβ1-3[Fucα1-4]GlcNAcβ1-R</td>
</tr>
<tr>
<td>Le^b</td>
<td>Lewis antigen: Fucα1-2Galβ1-3[Fucα1-4]GlcNAcβ1-R</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------------------------------</td>
</tr>
<tr>
<td>Man</td>
<td>Mannose</td>
</tr>
<tr>
<td>MBP</td>
<td>Mannose binding protein</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-[N-morpholino]-propanesulphonic acid</td>
</tr>
<tr>
<td>MRL lpr/pr</td>
<td>MRL/Mp-lpr/lpr</td>
</tr>
<tr>
<td>MRL +/-</td>
<td>MRL/Mp-+/-</td>
</tr>
<tr>
<td>Neu5AC</td>
<td>N-acetyleneuraminic acid (sialic acid)</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotide</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>p58&lt;sup&gt;GTA&lt;/sup&gt;</td>
<td>β1,4-GalTase associated protein kinase</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood lymphocytes</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pITC</td>
<td>Phenol isothiocyanate</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PMFP</td>
<td>Permanent mixed-field polyagglutinability</td>
</tr>
<tr>
<td>PWM</td>
<td>Pokeweed mitogen</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RCAI</td>
<td>Ricinus communis agglutinin I</td>
</tr>
<tr>
<td>RER</td>
<td>Rough endoplasmal reticulum</td>
</tr>
<tr>
<td>RF</td>
<td>Rheumatoid factor</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>RPA</td>
<td>RNase protection assay</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>R-PE</td>
<td>R-phycoerythrin</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
</tbody>
</table>
Ser Serine
slgG Surface immunoglobulin G
SLE Systemic lupus erythematosus
SRBC Sheep red blood cell
SSC Sodium chloride, sodium citrate
SSPE Sodium chloride, sodium dihydrogen phosphate, EDTA
STE Sodium chloride, Tris, EDTA
T Polyoxyethylene sorbitan monolaurate (Tween 20)
TBE Tris, boric acid, EDTA
TCR T cell receptor
TE Tris, EDTA
TEMED Tetramethyl-ethylenediamine
Tf Thomsen-Friedenreich: Galβ1-3GalNAcα1-O-Ser/Thr
Thr Threonine
Tn GalNacα1-O-Ser/Thr
TNE Tris, sodium chloride, EDTA
TNF-α Tumour necrosis factor-α
Trp Tryptophan
TTP Thymidine triphosphate
Tyr Tyrosine
UDP Uridine diphosphate
UV Ultraviolet
VH Variable region of immunoglobulin heavy chain
VL Variable region of immunoglobulin light chain
VSV Vesicular stomatitis virus
VV Volume/volume
X-Gal 5-Bromo-4-chloro-3-indolyl-β-D-galactoside
ZP3 Zona pellucida 3
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CHAPTER 1

GENERAL INTRODUCTION
1.1 Rheumatoid arthritis and IgG

1.1.1 Rheumatoid arthritis

1.1.1.1 Human rheumatoid arthritis

Rheumatoid arthritis is a chronic systemic inflammatory disease of unknown aetiology with several features of autoimmunity. This debilitating disease primarily involves synovial tissues and can lead to erosion, deformity and destruction of the joints. It affects 0.5-5% of the population worldwide (Felson 1993) and occurs most often between the ages of 20 and 60 years of age, with the peak age of onset about 50 years and a female: male ratio of 2 or 3:1 (McCarty 1993). Historically there is evidence that it may be a relatively “recent” condition, clear accounts of the disease having been written only during the last two centuries (Dieppe and Rogers 1993).

The clinical heterogeneity of RA is well known; some cases are very mild and self-limiting, while others are seriously progressive leading to joint lesions and sometimes including extra-articular manifestations which can be life-threatening (Sany 1994). Extra-articular manifestations include subcutaneous nodules, fibrosing alveolitis, vasculitis of the skin (infarcts/ulcers), nerves (neuropathy) and eyes (scleritis), muscle atrophy, and Felty’s and Sjögren’s syndromes (Bacon 1993).

There is abundant evidence to indicate that RA is an immunologically driven disease. Both the histological appearance in the synovium and the serological changes point to intense immunological activity. The inflamed synovium in RA is characterised by a proliferation of the lining layer and formation of a pannus tissue where cells of the synovium penetrate and destroy the cartilage and bone (at the cartilage-synovial junction). In the sub-membrane area there is proliferation of vascular endothelial cells with perivascular infiltrates of polymorphs and large numbers of T-lymphocytes, mainly CD4⁺, in various stages of activation as seen from their expression of
activation markers such as HLA-DR. The aggregates of T cells are usually associated with dendritic cells and macrophages; clumps of plasma cells are frequently observed and occasionally even secondary follicles with germinal centres are present (Roitt 1994, Zvaifler 1993). The appearance come to resemble an immunologically active lymph node with the synthesis of immunoglobulins by the synovial tissue B cells/plasma cells ranking with that of a stimulated lymph node (Smiley et al 1968). The synovial lining cells and the majority of cells of the infiltrative pannus show a high and induced expression of class II antigens (Cush and Lipsky 1988, Burmester et al 1987).

The mechanisms of the joint damage may be inferred from the immunopathology of the disease. The antigen (still unknown) is presented by class II positive cells to CD4+ lymphocytes. These are activated and secrete GM-CSF and TNF-α which recruit more CD4+ cells, B-cells and polymorphs, and stimulate macrophages to increase their production of cytokines such as IL-1, IL-6 and TNF-α. B cells, under the influence of growth factors, differentiate into plasma cells and secrete rheumatoid factors which form immune complexes. These complexes activate the complement cascade, resulting in an increase in capillary permeability and influx of serum proteins and cellular blood elements into the joint fluid. Immune complexes are also phagocytosed by macrophages and polymorphs, leading to further activation. The resultant release of lysosomal proteinases (including collagenase, elastase, cathepsins) and the production of reactive oxygen intermediates and arachidonic acid metabolites contribute to tissue destruction (Zvaifler 1993, Brostoff et al 1991).

RA has a complex, multifactorial aetiology. All the available evidence points towards a complex interaction between genetic, hormonal and environmental factors in determining both susceptibility to RA and its ultimate severity. Differences in concordance rates among monozygotic and dizygotic twins reveal a great deal about the relative importance of genetic and non-genetic
factors in complex disorders. From twin studies, it is quite clear that the concordance rates for the disease in identical twins (12-30%) are very low and, therefore, there must be an important non-genetic component to RA. On the other hand, the importance of the genetic contribution may be inferred from at least fourfold greater concordance for the disease in monozygotic than in dizygotic twins (2-3% concordance rate) in most twin studies conducted (Lawrence 1970, Aho et al 1986).

The genetic contribution to RA can be explained, but only in part, by gene(s) in the HLA region. The relative risk of developing RA is several times greater in individuals who inherit HLA-DR1 or -DR4, specifically the subtypes Dw4, Dw14, Dw15, and Dw16. Molecular studies have shown that these DR alleles share an oligonucleotide sequence corresponding to five amino acids in the third hypervariable region of the DRβ1 chain. This shared epitope may account for most of the HLA predisposition (Gao et al 1990, Nepom et al 1989, Wordsworth 1990, Gregerson et al 1986, Wilkens et al 1991). Other genes outside the HLA region that may contribute to the inherited disease susceptibility include loci on chromosome 14, including the α-1 antitrypsin gene (Ollier et al 1988, Sanders et al 1986, Sanders et al 1988), and possibly the T cell receptor genes (Olee et al 1991).

The search for an infectious aetiology of RA continues. A large number of infectious microorganisms have been proposed to be associated with RA. These include several strains of bacteria such as Proteus mirabilis (Ebringer et al 1985), slow-growing bacteria (Rook and Stanford 1992), mycoplasma and several viruses such as EBV and rubella (Philips and Inman 1993). The claims on implicating specific microbial agents in RA have been interpreted with caution, as some of these organisms may be part of the traffic through the joint rather than the initial trigger, or may have been the result of contamination of cell lines grown in vitro, as is probably the case with mycoplasma. In other cases, abnormal immune responses to several microbial agents such as EBV was thought to be due to defective
immunoregulation by T cells, in other words, the altered immune response to EBV was interpreted not as the cause but as a result of the disease. Some evidence for the involvement of microbial agents in RA, such as mycobacterial heat shock protein and EBV, suggests molecular mimicry (i.e. cross-reactive antigens) as the mechanism for any role that the microbe may have in RA. At present, no firm evidence overwhelmingly implicates a specific microbial agent as a trigger of RA, and the studies directly or indirectly involving microbes in RA all require confirmation (Philips and Inman 1993).

The importance of hormones in RA is shown by the observations that: the disease is more common in women; the onset of disease is usually after the menarche and before the menopause; RA frequently goes into remission during pregnancy; after pregnancy, the risk of incident disease is increased, and the pre-existing disease is likely to show a flare-up (Ostensen and Husby 1983); women with RA are more likely to have low fertility rates than the age-matched controls (Spector et al 1990); and men with RA appear to have low circulating testosterone levels (Cutolo et al 1988). There is conflicting data regarding the effect of exogenous hormone use (oral contraceptives or replacement oestrogens) on the incident and severity of RA (Felson 1993). Other factors such as stress (Adler 1985) and diet (Buchanan et al 1991) have also been considered in the precipitation of RA.

1.1.1.2 Animal models of arthritis

Animal models of RA have played a valuable role in arthritis research and have made major contributions to the understanding of the basic mechanisms of autoimmunity and inflammation involved in the pathogenesis of joint disease. Although nearly all of the experimental animal models of arthritis share some similarities with human RA, none of them constantly reflects the arthritic process and the histological, genetic, immunological and systemic features as found in the human disease.
a) Experimentally-Induced Arthritis

Arthritis may be induced by using infectious agents (Kaklamanis 1992) or their components such as streptococcal cell wall-induced arthritis (Van Den Broek et al 1990, Case et al 1989) and adjuvant arthritis (Knight et al 1992 [mice], Pearson et al 1956 [rats]). The major protein constituents of cartilage have also been used to induce type II collagen arthritis both in rats (Trentham et al 1977) and in mice (Holmdahl et al 1990) and proteoglycan-induced arthritis (Dayer et al 1990, Mikecz et al 1990) in mice. In another case, injection of pristane (mineral oil) into mice has resulted in an experimental autoimmune disease with similarities to RA (Wooley et al 1989). In addition, intra-articular immune reactions have been noted following systemic immunization (antigen-induced arthritis; Schalkwijk et al 1990, Staite et al 1990).

b) Spontaneous arthritis

Naturally developing arthropathies occur in domestic pets and livestock (Bennett 1986), but their value is limited when the requirements of a model system include reproducibility and a known time of onset. Spontaneous arthritis is also observed in several strains of mice including the MRL lpr/lpr, DBA/1, NZB and Biozzi mice (Hang et al 1982, Nordling et al 1992, Nakamura et al 1991, Bouvet et al 1990, respectively). These forms of arthritis usually develop more slowly than the induced models except for MRL lpr/lpr, which shows signs of an arthritis by three months of age (Hang et al 1982). Spontaneously developing arthritis in normal mice have been shown to be influenced by the microbial environment, stress and hormonal levels (Waites and Whyte 1987, Holmdahl et al 1992).

MRL lpr/lpr

The MRL strain was developed in 1976 by Murphy and Roths at the Jackson Laboratory (Murphy and Roths 1979). Studies of the MRL/Mp-lpr/lpr (MRL lpr/lpr) mouse started as a description of a mutant mouse with enlarged lymph nodes and a lupus-like autoimmune disease. Homozygotes display
severe lymphadenopathy, splenomegaly and multiple autoantibodies. Hypergammaglobulinaemia and immune complex disease begins by 8 weeks of age and animals die (50% mortality in most colonies) within 17-22 weeks of age, usually from renal failure and vasculitis. MRL lpr/lpr mice develop an erosive synovitis, although the incidence and extent varies from colony to colony. The arthritis is usually detectable at 3-4 months of age by a characteristic histopathology, which consists of early proliferation of synovial cells followed by lymphoid pannus formation. By 5-6 months of age, significant joint pathology consisting of pannus induced erosion and articular cartilage destruction has occurred. Rheumatoid factors of the IgM and IgG subclasses are frequently detectable in the serum (Hang et al 1982, O'Sullivan et al 1985, Theofilopoulos and Dixon 1985, Koopman and Gay 1988).

The congenic MRL/Mp-+/+ (MRL +/+ ) differs from the MRL lpr/lpr mice by lacking the lymphoproliferative “lpr” gene (Theofilopoulos and Dixon 1985). The “lymphoproliferation” has been traced to an insertion of a retrotransposon element, Etn, in the fas antigen gene, causing a defect in fas-mediated apoptosis (Watanabe-Fukunaga et al 1992). This is thought to be the cause of a massive accumulation of an abnormal population of B220⁺, CD4⁻, CD8⁻ (“double negative”, DN) T cells in the peripheral lymph nodes and to a lesser extent in the spleen of MRL lpr/lpr mice. Further studies established that this T cell subset displays deficient activation in response to signals initiated by the antigen-specific T cell receptor (TCR)/CD3 complex (Altman et al 1994).

The spontaneous production of autoantibodies reflects the abnormal function of B cells in lpr mice (Cohen and Eisenberg 1991). Antibodies to chromatin (histones), ss-DNA and mouse IgG are found in lpr strains with various background genes. These specific autoantibodies are accompanied by a polyclonal increase in serum immunoglobulin. Spleen cells from MRL lpr/lpr mice have increased numbers of Ig-secreting cells. A non-specific polyclonal
activation has been demonstrated at an early age, however, as disease progresses in the MRL/lpr/lpr mouse, certain specificities, such as anti-Sm, show a disproportionately increased number of antibody-producing cells, which suggests that an antigen-specific mechanism has supervened. C57BL/6 Lpr/lpr B cells have also been shown to be resistant to tolerisation and demonstrate abnormally rapid capping of their immunoglobulin receptors, even in lpr mice that lack T cells (C57BL/lpr-nu/nu). On the other hand, their autoantibody production in response to a chronic graft-versus-host reaction is no different from that of C57BL/6 +/+ B cells (Eisenberg et al 1994). Defects in other cell types from MRL lpr/lpr strain mice have also been reported, including an enhanced state of activation of peritoneal macrophages (Dangvu et al 1987, Rokutan et al 1988) and decreased responsiveness of neutrophils (Gresham et al 1991).

1.1.2 Immunoglobulins and rheumatoid arthritis

Over 50 years ago, Waaler (1940) and subsequently Rose and colleagues (1948) discovered elevated levels of serum rheumatoid factor (RF) in patients with RA. Rheumatoid factors are defined as autoantibodies reactive with epitopes in the Fc portion of IgG. Although initially believed to be specific for RA, these autoantibodies may occur in patients with other connective tissue diseases such as systemic lupus erythematosus (SLE) and Sjögren's syndrome, in infectious diseases, such as rubella, leprosy, and malaria, and in the sera of normal individuals. Nonetheless, the presence of significantly elevated levels of serum RF is both characteristic of RA and provides a marker for increased susceptibility to developing RA (Schrohenloher and Koopman 1993). RFs associated with RA are distinctive in that they are of all immunoglobulin isotypes (not just IgM, indicating T-cell participation in antibody class-switching), have higher avidity for human IgG than for rabbit IgG, use the human germline heavy-chain variable region gene VhIII more frequently than other Vh genes, and light chains from multiple families (Hay et al 1991). IgG RFs in RA are somatically mutated (in comparison to
monoclonal RFs which use unmutated germline Ig genes) suggesting that the B cells producing them have been stimulated either with specific antigens or other activation signals such as cytokines (Hay et al 1991). The synovial membrane has been shown to be a prime site for RF production, with plasma cells containing all five Ig classes but showing a bias towards particular IgG subclasses (Munthe and Natvig 1972). Analysis of immunoglobulins secreted by hybridomas derived from rheumatoid synovia also indicated that RA B cells are under a selective influence, as shown by over-representation of certain subclasses and light chains of IgG (Brown et al 1990).

There is substantial evidence that RFs contribute to tissue injury in RA. Immune complexes in sera, synovial fluids, and within polymorphonuclear leukocytes of patients with RA have been shown to consist mainly of IgG, IgM, and complement components, suggesting that IgG itself may be the antigen (i.e. IgG is both antigen and antibody), either as self-associating IgG RFs or as IgG altered by defective glycosylation (discussed below). These complexes can in turn activate the complement system resulting in tissue injury as described earlier, interact with cell surface receptors, as in activation of natural killer cells by cross-linking their Fcγ receptors (Hendrich et al 1991), or be deposited in tissues. In addition, disease severity and activity in RA tend to correlate with levels of RF (Alarcon et al 1990, Allen et al 1981).

A variety of autoantibodies other than RFs have been described in patients with RA. These include antibodies to cellular antigens such as histones, single-stranded DNA, nuclear antigens, perinuclear factor, ribosomal proteins, and antibodies to cytoskeletal components such as intermediate filaments (Baum et al 1993). Anti-collagen II antibodies have been eluted from rheumatoid cartilage (Jasin 1985) and collagen-anticollagen immune complexes have been detected in RA synovial fluid (Clague and Moore 1984). Antibodies to other cartilage components such as the minor cartilage collagens (type V, VI, and IX) have also been reported (Jasin and Taurog 1991, Morgan et al 1989).
B-cells from patients with RA show evidence of increased activation in culture and secrete immunoglobulin and RFs spontaneously, whereas B-cells obtained from healthy individuals will only do so after stimulation with polyclonal activators, such as PWM and EBV (Dalal et al. 1990, Koopman and Schrohenloher 1980, Slaughter et al. 1978). The number of peripheral blood B cells in RA is within the normal range (Holoshitz et al. 1989), however, increased numbers of B-cells expressing the CD5^ marker have been detected in the circulation of patients with RA (Plater-Zyberk et al. 1985, Taniguchi et al. 1987, Youinou et al. 1990). CD5^ B-cells from both normal individuals and patients with RA produce a variety of autoantibodies including RF when stimulated in vitro (Casali et al. 1987). While autoantibodies produced by CD5^ B cells from healthy individuals exhibit low affinity and polyreactivity, those from patients with RA also produce higher affinity, monoreactive RF (Nakamura et al. 1988, Burastero et al. 1988). Whether the CD5 marker on B cells represents a distinct B-cell lineage or is just an activation antigen enhanced in RA (Werner-Favre et al. 1989) consistent with increased B cell expression of other activation markers such as HLA-DR molecules and CD21 (Hildebrandt et al. 1988) remains to be clarified.

1.2 Defects in IgG glycosylation in rheumatoid arthritis

1.2.1 IgG structure
IgG comprises about 80% of the antibodies in serum. In 1959 Rodney Porter showed that the plant protease papain splits the immunoglobulin molecule to two identical fragments, each with a single combining site for antigen (Fab, fragment antigen binding) and a third fragment which lacks the ability to bind antigen and is termed Fc (fragment crystallisable, complement and cell binding). In 1962, he proposed that the immunoglobulin molecule is a glycoprotein composed of two identical heavy polypeptide chains and two identical light polypeptide chains. These are crosslinked by interchain
disulphide bonds and stabilised by non-covalent interactions. Both heavy and light chains are folded into globular domains containing β-pleated sheets stabilised by intra-chain disulphide bonds, a structural motif known as the immunoglobulin fold. The amino-terminal domains of one heavy and one light chain together form an antigen binding site, and these domains are highly variable between different antibodies (V domains). The remaining domains are less variable and are referred to as constant (C) domains. The constant domains of the heavy chain in the IgG molecule are designated C\textsubscript{H}1, C\textsubscript{H}2 and C\textsubscript{H}3. Some of these constant domains (Fc) are responsible for most of the effector functions, such as binding to Fc receptors, expressed on macrophages and monocytes, and binding to complement components.

Porter also showed that the major glycosylation site of IgG is within the Fc portion. Now it is known that an integral feature of all normal immunoglobulins is the conserved N-linked oligosaccharides in the C\textsubscript{H}2 domain. The glycosylation of the C\textsubscript{H}2 domain has been conserved throughout evolution, suggesting an important role for the oligosaccharide. Oligosaccharide units have also been reported to be present on the light chain, presumably on the variable region only, since no potential glycosylation site is present on the constant region of the light chain. Approximately 25% of the Fab fragments and 15% of the light chains isolated from human myeloma proteins contain an N-linked oligosaccharide chain (Wright and Morrison 1993).

An IgG molecule contains, on average, 2.8 N-linked oligosaccharides, of which two are invariably located in the C\textsubscript{H}2 domain of the Fc at the conserved N-glycosylation site of asparagine 297. Exoglycosidase sequencing of oligosaccharides chemically released from serum IgG revealed that these sugar chains are either sialylated or non-sialylated biantennary complex-type in which the presence or absence of fucose, of bisecting N-acetylgalactosamine and of galactose residues give rise to extremely high microheterogeneity (Parekh et al 1985). An exception to this is mouse IgG which does not contain bisected oligosaccharides (Mizuochi et al 1987). The
basic structure of these biantennary complex-type oligosaccharide chains is shown in Figure 1.1:

\[
\pm \text{Neu5Ac}_2 \rightarrow 6 \pm \text{Gal} \beta 1 \rightarrow 4 \text{GlcNAc} \beta 1 \rightarrow 2 \text{Man} \alpha 1 \rightarrow 6 \pm \text{Fuc} \alpha 1 \\
\pm \text{GlcNAc} \beta 1 \rightarrow 4 \text{Man} \beta 1 \rightarrow 4 \text{GlcNAc} \beta 1 \rightarrow 4 \text{GlcNAc} \rightarrow \text{Asn} \\
\pm \text{Neu5Ac}_2 \rightarrow 6 \pm \text{Gal} \beta 1 \rightarrow 4 \text{GlcNAc} \beta 1 \rightarrow 2 \text{Man} \alpha 1 \rightarrow 3
\]

**Figure 1.1** The largest N-linked oligosaccharide chain of human IgG. The presence or absence of glycan residues leads to heterogeneity. Neu5Ac: N-acetylneuraminic acid, Gal: galactose, GlcNAc: N-acetylgalactosamine, Man: mannose and Fuc: fucose.

Thus, 32 different biantennary oligosaccharides are found to be associated with total human serum IgG (Parekh *et al* 1985). The large number of different glycoforms associated with IgG is not the result of studying a polyclonal population, since a similar heterogeneity is found upon analysis of myeloma and hybridoma IgG (Rademacher *et al* 1986). Despite this extremely high microheterogeneity, the molar ratio of each of these oligosaccharides included in the IgG samples obtained from the sera of healthy individuals is quite constant (Mizuochi *et al* 1982).

There are strong (non-covalent) trans-interactions between the protein domains: such as V\(_L\) interacting with V\(_H\), C\(_L\) with C\(_H1\), and two C\(_H3\) domains interacting with each other. However, no interaction is seen between the two C\(_H2\) domains. Instead the sugar chains are interposed between them (Sutton and Philips 1983, Deisenhofer 1981). Crystallographic studies of IgG Fc fragments have shown that the 1-6 arms from each biantennary oligosaccharide are directed towards the protein surface where the terminal sialic acid-galactose lies in a special lectin-like pocket while the 1-3 arms, one of which always lacks galactose, appears to interact with the trimannose core of the opposing oligosaccharide, thus bridging the two C\(_H2\) domains (Parekh *et al* 1985, Opdenakker *et al* 1993).
1.2.2 Biochemistry of glycosylation

1.2.2.1 Classification of protein-linked oligosaccharides

Protein-bound oligosaccharides are classified according to the type of linkage between the sugar and the polypeptide backbone. Those that are called mucin-type or O-linked glycans generally contain an N-acetylgalactosamine residue, at their reducing termini, linked to the hydroxyl group of either a serine or a threonine residue of a polypeptide. Recently novel O-glycosidic linkages have been discovered involving other sugars such as glucose, galactose and N-acetylglucosamine and amino acids tyrosine, hydroxylysine and hydroxyproline. Asparagine-linked or N-linked glycans contain an N-acetylglucosamine residue at their reducing termini and are linked to the amide group of an asparagine residue of a polypeptide. During recent years, several new asparagine-linked monosaccharides have been discovered, mainly in bacterial glycoproteins, namely glucose, N-acetylgalactosamine and L-rhamnose. A third type of linkage to proteins has been found for an increasing number of cell surface proteins, which are known to be inserted into the lipid bilayer via a glycoprophosphatidylinositol (GPI) anchor. Only six amino acids serve as a GPI attachment site: Asp, Asn, Gly, Ala and Ser. In this type of linkage, the carbohydrate is attached to the C-terminal amino acid of the protein via ethanolamine phosphate (Lis and Sharon 1993, Dwek 1995).

1.2.2.2 N-linked oligosaccharides; structure and biosynthesis

All N-linked glycans share the common core structure Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc which is called the “trimannosyl core”. On the basis of the structure and the location of the glycan residues added to the trimannosyl core, N-linked glycans are further classified into four subgroups: (i) High mannose N-glycans contain only mannose residues attached to the core. (ii) Complex N-glycans have “antennae” or branches attached to the core. These branches are initiated by the action of four mammalian GlcNAc-
transferases and may be further elongated by the addition of galactose, N-acetylgalactosamine, fucose, sialic acid and sulphate. The number of antennae in mammals ranges from two (bianternary) to four (tetraantennary).

(iii) Hybrid N-glycans have only mannose residues on the Manα1-6 arm of the core and one or two antennae on the Manα1-3 arm. (iv) Poly-N-acetyllactosamine N-glycans contain repeating units of (Galβ1-4GlcNAcβ1-3) attached to the core. This repeating structure may be further branched by the action of β6-GlcNAc-transferase. All N-glycans except the high mannose type may be “bisected” by a GlcNAc residue attached in β1-4 linkage to the β-linked mannose of the core (Kornfeld and Kornfeld 1985).

The biosynthesis of N-glycans begins in the rough endoplasmic reticulum (ER) as a co-translational event in contrast to other glycosylations (O-glycans or GPI addition) which are post-translational. The membrane-bound oligosaccharyl-transferase enzyme catalyses the en bloc addition of a large oligosaccharide (Glc₃Man₉GlcNAc₂) from a preformed lipid (dolichol pyrophosphate) donor, to an Asn residue in the polypeptide. The Asn must be in an Asn-X-Ser/Thr sequon (consensus sequences for glycosylation), where X is any amino acid except Proline. This is followed by the removal of three glucose and four mannose residues within the lumen of the ER and Golgi apparatus due to the processing actions of specific α-glucosidases and α-mannosidases. The product of this “trimming” reaction is the structure which serves as the starting point for the synthesis of all complex and hybrid N-glycans. Oligosaccharide processing continues in the compartments of the Golgi, with a variety of exoglycosidase and glycosyltransferase reactions leading to the generation of “complex-type” oligosaccharide structures. All of the terminal-region sugars are added in the trans Golgi by a series of glycosyltransferases that act in a rigidly determined sequence, thus the addition of outer chain galactose and sialic acid residues are catalysed by galactosyl- and sialyltransferases. The newly synthesised glycoproteins then exit the Golgi and are transported to their final destination (Snider 1984, Kornfeld and Kornfeld 1985).
1.2.2.3 Regulation of oligosaccharide synthesis

Glycoproteins can have one to over twenty sites glycosylated. Site analysis has shown that modification of the N-linked oligosaccharide structure is often highly specific for each individual glycosylation site on the protein. Each site usually has a set of closely related oligosaccharides (site heterogeneity), giving rise to different glycoforms of a glycoprotein. The proportions of these glycoforms, however, remain constant and reproducible in particular physiological states, indicating that the site variation or "microheterogeneity" cannot just be the result of random variation. For example, the consistency in the ratio of oligosaccharide variants of IgG-Fc preparations obtained from the sera of healthy individuals indicates that this glycosylation process must be regulated by a very potent control system, although the mechanism apart from identification of the constituting elements, is still unknown. In addition to these enigmas, not all the potential sites in a glycoprotein are glycosylated. It is estimated that between 10% and 30% of potential glycosylation sites are not occupied (Opdenakker et al 1993, Dwek 1995).

There are several factors to consider in understanding the control of protein glycosylation. Firstly, the general characteristics of the glycoprotein being processed: the primary peptide structure determines the number and location of potential glycosylation sites and the overall three-dimensional protein conformation determines the extent and type of glycosylation based on the position and accessibility of the glycosylation site in the protein. For example, sequons close to the amino or carboxyl-termini are generally less efficiently glycosylated (Opdenakker 1993). Secondly, the available repertoire of the glycosylation processing enzymes in a particular cell type (species-specific, tissue- and cell-type specific glycosylation): the type, concentration, activity and compartmentalization of glycosidases and glycosyltransferases present in a cell have a major role in determining the extent and type of glycosylation. Thirdly, the local environment and conformation at the individual glycosylation sites (site-specific glycosylation, Baenziger 1994): the pH of the local environment, the availability of necessary metabolites such as divalent
cations (Mn\textsuperscript{2+} and Ca\textsuperscript{2+}), pyrophosphatases and phospholipid, and the local protein conformation influence the glycosylation event (Dwek 1995, Goochee and Monica 1990). In particular folding or packaging of the protein that involves disulphide bridges may hinder glycosylation (Opdenakker 1993). The complexity of the glycosylation process is only fully understood when the interactions between these factors is taken into consideration. The competition between processing enzymes for a common substrate usually determines the route of the biosynthetic pathway. Sometimes, the product of a transferase reaction serves as a "stop" signal in the synthetic pathway, e.g. insertion of a bisecting GlcNAc prevents the actions of α3/6-mannosidase II, core α6-fucosyltransferase and of GlcNAc-transferases II, IV and V, thereby effectively halting further branching (Schachter 1986, Brockhausen et al 1988). Interference with vesicle transport between the ER and subsequent Golgi compartments also affects glycosylation. Some glycoproteins are subject to sorting mechanisms that regulate their direction into regulated or constitutive pathways. Environmental agents, such as amines and ionophores, which affect intracellular membrane fusions can disrupt intracellular transport and sorting mechanisms. Glucocorticoids regulate the intracellular trafficking of some glycoproteins. There is evidence that the addition of galactose residues occurs distal to, or at, the actual hormone-induced regulatory step. In the absence of glucocorticoids, glycoproteins with a glucocorticoid sorting signal are inefficiently galactosylated (Haffar et al 1988). Competition between the rate of folding, as determined by the polypeptide sequence, and the addition of dolichol-linked precursor/oligosaccharides may also affect glycosylation (Opdenakker 1993).

1.2.2.4 Biological roles of oligosaccharides

Some general observations about glycoproteins point to an important role of oligosaccharides in modifying the structure and/or function of a protein. These include the enormous structural diversity of oligosaccharides, the widespread occurrence of glycoproteins, the conservation of some
carbohydrate units of glycoproteins throughout evolution and the fact that oligosaccharide structures of glycoproteins sometimes undergo dramatic changes with differentiation and development and also in physiological and pathological processes. The ability of the oligosaccharides to modulate the structure, dynamics and physico-chemical properties of the proteins to which they are covalently bound is well documented. These include facilitation of the proper folding, stabilisation of the conformation and protection against proteolysis. There is also evidence that some oligosaccharides modify solubility, electrical charge, viscosity in solution and thermal stability. Glycosylation may also modulate the functional activity of enzymes and hormones, as is the case with tissue plasminogen activator and erythropoietin (Dube et al 1983, Wittwer et al 1989).

Additionally, there is increasing evidence that oligosaccharides bound to proteins or lipids act as recognition determinants in cell-cell and cell-molecule interactions. Recognition of oligosaccharide sequences by certain receptors (hepatic asialoglycoprotein receptor, serum mannose binding protein and the mannose receptor on some macrophages and hepatic endothelial cells) can result in clearance of the glycoconjugate or even the whole cell (e.g. pathogens or transformed cells) from the circulation, thereby controlling protein and cell half-life (Ashwell and Hartford 1982, Drickamer 1988). Certain oligosaccharides can act as specific receptors for a variety of viruses, bacteria and parasites (binding of terminal sialic acids to haemagglutinins from influenza A virus), thereby acting as attachment sites in the initial stages of infection, whilst others are receptors for plant and bacterial toxins (e.g. G\textsubscript{M\textsubscript{1}} gangliosides binds to cholera toxin). Some carbohydrate structures serve as antigens for autoimmune or alloimmune reactions (e.g. polylactosamine chains on red blood cells (i antigen), Tn antigen and the blood group ABO antigens) (Lis and Sharon 1993, Varki 1993).

Oligosaccharides are also involved in leukocyte migration and recruitment into sites of inflammation. The selectin family of receptor proteins mediate the
adhesion of leukocytes to endothelial cells (L-selectin, CD62L), the recognition of leukocytes by stimulated or wounded endothelium (E-selectin, CD62E) and the interactions of activated platelets or endothelium with leukocytes (P-selectin, CD62P). In each case, the minimal oligosaccharide sequences recognized appear to be sialylated fucosylated sugar chains, such as Sialyl Lewis x and Sialyl Lewis a (Foxall et al 1992).

A role in intracellular trafficking of proteins has also been proposed for oligosaccharide sequences. The best example is binding of the mannose-6-phosphate receptor in the Golgi apparatus to the high mannose-type oligosaccharides on lysosomal enzymes, which then initiates the sorting events that targets the enzymes to the lysosomes (Kornfeld 1987).

Specific recognition of carbohydrate structures has also been documented in fertilisation. It has been suggested that β1,4-galactosyltransferase (β1,4-GalTase) enzyme, which is present on sperm heads, binds to oligosaccharides on the ZP3 glycoprotein of the zona pellucida of the egg. After fertilisation, the loss of sperm receptor activity due to modification of ZP3 prevents additional sperm binding to the zona pellucida (Florman and Wasserman 1985, Miller et al 1992).

Although various studies demonstrate that the oligosaccharide units of glycoconjugates play diverse and crucial roles, no generalisation can be made so far. For example, the removal of oligosaccharides from glycoproteins may have detrimental consequences for one glycoprotein and no effect on another one. Therefore, each glycoprotein must be evaluated individually to determine the contribution of the oligosaccharides to its physical properties and biological functions (Varki 1993).
1.2.3 Abnormalities in IgG oligosaccharides

Although some of the changes detected in the distribution of glycoforms of IgG have been found to be part of a natural physiological change, there are also changes which have been associated with disease. An increasing number of diseases, with or without a known "genetic" defect in glycosylation, are now being found to be associated with abnormalities in the oligosaccharide moieties of glycoproteins (Varki 1993). Amongst these are tumour-associated carbohydrate changes (Singhal and Hakomori 1990), alterations in the serum glycoprotein $\alpha_2$-macroglobulin in RA and SLE (Silvestrini et al 1989) and changes in the acute phase protein $\alpha_1$-acid glycoprotein in RA, SLE and ankylosing spondylitis (Mackiewics et al 1987, Mackiewicz et al 1989a). Genetic disorders affecting glycosylation will be discussed later in this chapter. The growing list of diseases associated with abnormalities in glycosylation shows the important but neglected role of glycobiology in disease.

1.2.3.1 Agalactosyl IgG

a) Human agalactosyl IgG or "G0"

Structural anomalies in serum IgG were reported for the first time in 1974 when Johnson and colleagues noted differences in the circular dichroism spectra (a spectropolarimetric method of studying conformational changes in macromolecules) of serum IgG in health and rheumatoid disease (Johnson et al 1974). The same group also showed that these changes were present in serum IgG from tuberculosis and SLE patients. Then in 1975 Mullinax and Mullinax reported low levels of IgG galactose in patients with RA and SLE. The defect was localised to the Fc region and an enzymatic origin was suggested for this deficiency, although an increase in $\beta$-galactosidase enzyme was ruled out (Mullinax and Hymes 1976). These findings were largely ignored until 1985, when two groups in Oxford and Tokyo jointly analysed ~1,400 oligosaccharides from IgG samples isolated from patients with RA and primary osteoarthritis and normal individuals (Parekh et al 1985).
They too reported an altered glycosylation pattern of serum IgG in the patients, as a “shift” in the population of IgG molecules towards those carrying complex oligosaccharides, one or both of whose arms terminate in GlcNAc. Thus, the IgG glycoforms were defined in terms of the terminal sugar residues in the antennae of Fc complex oligosaccharide, as di-galactosyl (G2), mono-galactosyl (G1) and agalactosyl (G0) oligosaccharides (Fig. 1.2).

Gal   Gal   Gal
|     |     |     |
GlcNAc GlcNAc GlcNAc

“G2”   “G1”   “G0”

Figure 1.2  The three possible terminal sugar combinations of the IgG Fc oligosaccharide in relation to the presence or absence of galactose. Each sugar has either two terminal galactose (Gal) residues “G2”, one Gal and one N-acetylglucosamine (GlcNAc) “G1”, or two terminal GlcNAcs “G0”.

Then in 1988, it was shown that the galactosylation of IgG oligosaccharide changes as a parabolic function of age in that the relative incidence of agalactosyl IgG decreases from birth to a minimum at 25 years of age and then increases with age (Parekh et al 1988a). Pregnancy was also found to be another physiological state associated with natural fluctuations in IgG galactosylation (Pekelharing et al 1988, Rook et al 1990).

When the age-related variation in IgG galactosylation was taken into account, the levels of agalactosyl IgG from sera of patients with osteoarthritis were found to lie within the normal range. Rheumatoid arthritis, however, was still linked to abnormal IgG galactosylation (Parekh et al 1985, Tomana et al 1988, Sumar et al 1990). Since then, an extensive disease screening search has shown that raised agalactosyl IgG levels exist in a limited number of other diseases including juvenile arthritis (Mizuochi et al 1982, Parekh et al 1988b, Bahr et al 1990, Sumar et al 1991a, Tsuchiya et al 1993), tuberculosis

Comparative analysis has shown that within the rheumatological and autoimmune disorders, the defect is quite disease-specific, with disorders such as primary SLE, ankylosing spondylitis and multiple sclerosis being found to be associated with normal levels of IgG G0 (Parekh et al 1989). Decreased galactosylation of IgG is not a marker of general inflammation either, since the majority of chronic infections such as leprosy, Klebsiella infection and AIDS or diseases associated with acute inflammation such as acute rheumatic fever and ulcerative colitis are found to have normal levels of galactose on IgG (Parekh et al 1989, Rademacher et al 1988). These observations have led to the suggestion that IgG G0 rises only when there is an acute-phase response superimposed on a pre-existing chronic inflammatory state (Rook et al 1989, Rook et al 1990). The IgG G0 level has been found to be higher in synovial fluid than in serum supporting the idea that agalactosyl IgG is produced in the joint in RA patients (Parekh et al 1989, Tsuchiya et al 1993, Bodman 1995).

Amongst the mechanisms proposed to cause the reduction of galactose residues on IgG are: a decrease in the activity of β1,4-GalTase enzyme as reported in RA B cells (Axford et al 1987, Furukawa et al 1990, Wilson et al 1993) and bone marrow myeloma cells (Nishiura et al 1990), and post-synthetic degradation of IgG by reactive oxygen intermediates (Griffiths and Lunec 1989) in which oxygen-free radicals generated by activated neutrophils and macrophages at inflammatory sites have been suggested to be responsible for raised agalactosyl IgG levels. In a study carried out by Sumar et al (1991b) spouses of patients with RA who were not affected by the
disease also showed raised levels of agalactosyl IgG. The authors suggested the involvement of an environmental factor (or perhaps sexually transmitted agents) in this deficiency.

b) Murine agalactosyl IgG

Following the reports of defective IgG galactosylation in human, Mizuochi and colleagues reported structural changes in the oligosaccharide chains of IgG in MRL $lpr/lpr$ mice. The defect was again found to be a significant reduction in the galactosylation of total serum IgG, in this case in that of MRL $lpr/lpr$ mice compared to MRL $+/+$ (Mizuochi et al 1990). In another study, MRL $lpr/lpr$ was compared to four other strains, MRL $+/+$, CBA/Ca, C57BL/6 and BALB/c (Bond et al 1990). Increased exposure of terminal GlcNAc was clearly detected in MRL $lpr/lpr$, confirming the previous finding. However, a rise in terminal galactose residues was also found in MRL $lpr/lpr$, suggesting that a second glycosylation site must be present on the IgG molecules. In 1994, Bodman et al showed that different mouse strains have characteristic “resting” levels of G0 which increase with age. They also showed that these increases can be enhanced by immunisation of arthritis-prone strains of mice with an adjuvant containing mycobacteria (Freund’s complete adjuvant, FCA). The finding of increased G0 levels in MRL $lpr/lpr$ mice compared to MRL $+/+$ and CBA/Ca mice was corroborated in this study. MRL $+/+$ mice were shown to have a higher resting level of serum G0 compared to that of CBA/Ca mice which increased significantly with age, but at a slower rate than in the MRL $lpr/lpr$ mice.

An increase in agalactosyl IgG was also described in two murine models of induced arthritis. Male DBA/1 mice injected with collagen type II were reported to have increased IgG G0 levels compared to controls (Jones et al 1992). Similarly, injection of pristane into male CBA/Ig$^h$ mice was shown to induce a rise in agalactosyl IgG which was followed by arthritis in mice with particularly high levels of IgG G0 (Rook et al 1991a). In both models, similarly
to in humans, pregnancy was reported to affect the severity of arthritis and the glycosylation status of IgG (Rook et al. 1991b, Thompson et al. 1992).

1.2.3.2 Other changes in IgG carbohydrates

Decreased levels of terminal sialic acid have been observed on IgG and IgM from patients with RA (Duc Dudon and Quash 1981, Casburn-Budd et al. 1992) and the IgG molecules with a lower content of terminal sialic acid have been suggested to be involved in rheumatoid factor activity. Decreased levels of IgG sialic acid have also been demonstrated in patients with primary Sjögren's syndrome (Youinou et al. 1992).

IgG purified from sera of patients with RA has been reported to show increased binding to concanavalin A (Con A), a lectin which mainly binds to mannose (Malaise et al. 1987). The increased number of accessible mannosyl residues on RA IgG is suggested to be responsible for the inhibition of the Fc receptor function of monocytes (Malaise et al. 1986). A myeloma IgG protein has also been reported to contain an oligosaccharide structure terminating in mannose as the nonreducing end of the α1-3 arm (Takahashi et al. 1987). Monoantennary oligosaccharides (terminating in mannose in one of the arms) were also isolated in another study from IgG2 and IgG3 proteins from sera of patients with multiple myeloma (Jefferis and Lund 1990). Studies by Jefferis et al. (1990) and Takahashi et al. (1987) also describe IgG molecules which have digalactosylated oligosaccharide moieties on both heavy chains.

Non-enzymatic glycation (covalent attachment of sugar molecules to amino groups of proteins) of IgG has been reported to be increased in diabetic patients and suggested to be linked to increased prevalence of infection in these patients. In vitro studies have shown that glycation decreases the ability of IgG to bind to protein A and to the C1q component of the complement (Dolhofer-Bliesner and Gerbitz 1990). In vitro glycation of IgG has also been reported to increase the vascular clearance rate of IgG in mice (Kennedy et al. 1993).
1.2.4 The effect of IgG glycosylation changes on IgG function

In vitro studies have been done on IgG molecules whose sugars were partially or completely removed by exo- or endoglycosidase digestion or on nonglycosylated IgGs produced by hybridoma cells in the presence of the antibiotic tunicamycin, which is an inhibitor of glycosylation. These studies showed that IgG molecules lacking the carbohydrate chain are deficient in complement fixation (Koide et al 1977, Nose and Wigzell 1983, Leatherbarrow et al 1985, Heyman et al 1985), antibody-dependent cellular cytotoxicity (Koide et al 1977, Lund et al 1990), binding to Fc receptors on monocytes/macrophages (Koide et al 1977, Nose and Wigzell 1983, Leatherbarrow et al 1985, Heyman et al 1985, Walker et al 1989, Lund et al 1990), and rapid elimination of antigen-antibody complexes from the circulation (Nose and Wigzell 1983, Heyman et al 1985). Furthermore, the non-glycosylated IgG molecules were found to be highly deficient in suppressing the immune response (Heyman et al 1985). Antigen (Williams et al 1973, Winkelhake et al 1980, Nose and Wigzell 1983, Heyman et al 1985) and protein A-binding capacity (Nose and Wigzell 1983, Leatherbarrow and Dwek 1983, Heyman et al 1985), however, did not change in the absence of the Fc-associated oligosaccharide. The capacity to trigger the phagocyte respiratory burst was also found to be unaffected in the absence of glycosylation (Pound et al 1993).

Wright and Morrison (1994) have used gene transfection techniques to produce mouse-human chimaeric antibodies in a Chinese hamster ovary cell line which is deficient in N-acetylglucosaminyltransferase I activity, thereby synthesising truncated sugars (oligomannosyl structures) not normally found on IgG. Although the resulting antibodies were assembled and secreted correctly and retained antigen specificity, they showed significantly reduced binding of C1q, a reduction in the affinity of binding to Fc receptors on U937 cells, and a greatly reduced in vivo half-life due to accelerated clearance from the circulation. Other studies have used amino acid substitution to investigate the importance of glycosylation in effector functions of IgG. Using this
technique Lund et al (1995) suggest that the hexasaccharide (GlcNAc\(_2\)Man\(_3\)GlcNAc) structure is sufficient for maintenence of Fc receptor recognition of IgG and replacement of amino acids making contact with the terminal galactose residue on the 1-6 arm do not affect recognition of IgG by Fc\(\gamma\) receptors. In another study replacement of Asn 297 by Ala was used to show that the aglycosyl mutant antibody had identical antigen binding ability to the wild-type antibody but a shorter biological half-life probably due to more rapid proteolytic digestion in the extravascular tissues and not in the circulation (Wawrzynczak et al 1992).

Reports on the function of IgG molecules which lack only the terminal galactose residues are fewer and more recent. In 1989, Tsuchiya and colleagues found a significant reduction in C1q binding and in Fc receptor-mediated binding to U937 cells in IgG molecules whose galactose residues were specifically removed by digestion with streptococcal \(\beta\)-galactosidase. Therefore, it was concluded that the galactosylation of IgG molecules must be particularly important for their immunological activities. In another study, anti-rhesus D IgG molecules with lower levels of terminal galactose showed reduced binging to Fc\(\gamma\)RI (on monocytes) and Fc\(\gamma\)RIII (on natural killer cells) leading to decreased antibody-dependent cellular cytotoxicity, but no change in binding to Fc\(\gamma\)RI on U937 cells in rosette formation assays (Kumpel et al 1994).

It has been postulated that the galactosylation defect in IgG could contribute to self-association of IgG complexes either by the terminal sialic acid-galactose on the Fab oligosaccharide of one IgG fitting into the lectin site left vacant by the lack of galactose on the Fc sugar of another IgG molecule, or by exposure of hidden antigenic determinants (Roitt et al 1988). Increased levels of terminal GlcNAc has been detected in immune complexes from patients with RA compared to that of soluble IgG from the same patients, suggesting that changes in IgG glycosylation are associated with immune complex formation (Bond et al 1995). It is proposed that anti-Fc (RF) can
block feedback immunosuppression by preventing the binding of immunoglobulin to Fc receptors. Such a property has already been shown for deglycosylated IgG as mentioned before (Heyman et al 1985). The consequence could be increased levels of autoantibodies ultimately leading to tissue damage (Rademacher 1991).

IgG galactose deficiency has been reported to occur in patients with RA prior to the onset of the disease (Tomana et al 1994). The levels of agalactosyl IgG were shown to be of diagnostic value in differentiating individuals with early synovitis who developed RA (Young et al 1991). In addition, an elevated percentage of serum agalactosyl IgG was shown to be an important prognostic marker for human RA (Van Zeben et al 1993, Van Zeben et al 1994). These studies, however, do not provide direct evidence that agalactosyl IgG plays a role in disease pathogenesis.

More direct evidence for a link between agalactosyl IgG and pathogenicity comes from studies done by Rademacher and colleagues (1994). They demonstrated that passive transfer of collagen-induced arthritis to T-cell primed mice can be enhanced when the IgG containing autoantibodies to type II collagen are treated with β-galactosidase before injection. Therefore, nonpathogenic doses of autoantibodies can be made pathogenic by altering their glycosylation state.

Evidence for a link between agalactosyl IgG and initiation of inflammation came from nuclear magnetic resonance (NMR) studies and X-ray data which showed that the terminal GlcNAc residues became exposed and accessible to the collagenous lectin mannose-binding protein (MBP) only in IgG molecules in which the Fc oligosaccharide lacked galactose. It was also demonstrated that multiple presentation of IgG G0 glycoforms to MBP resulted in activation of complement. This was suggested to contribute to the chronic inflammation of the synovial membrane in the affected joint in patients with RA (Malhotra et al 1995).
1.3 β1,4-galactosyltransferase enzyme

1.3.1 General enzymology and background

UDP-galactose: N-acetylglucosamine β1-4 galactosyltransferase (β1,4-GalTase, EC 2.4.1.38) belongs to a functional family of highly specialised enzymes, termed the glycosyltransferases, that participate coordinately in the biosynthesis of the carbohydrate moieties of glycoconjugates. Glycosyltransferases transfer sugar residues from an activated donor substrate, usually a nucleotide sugar, to a growing carbohydrate side chain of a glycoprotein or a glycolipid. These enzymes are highly specific with regard to the donor and acceptor substrates and to the type of linkage they form. It is estimated that there must be a few hundred distinct glycosyltransferases to account for the known carbohydrate structures on glycoconjugates (Natsuka and Lowe 1994). Amongst these glycosyltransferases, β1,4-GalTase is the most extensively studied to date.

β1,4-GalTase was initially discovered in particulate preparations from bovine and guinea pig mammary glands, when for the first time the reaction for lactose synthesis was described (Watkins and Hassid 1962). The biosynthesis of lactose was shown to involve the transfer of galactose from UDP-galactose to glucose to form lactose. In 1964 Babad and Hassid purified the enzyme from bovine milk and showed that it would catalyse the transfer of galactose from UDP-galactose to N-acetylglucosamine at about 25% the rate of transfer to glucose (Babad and Hassid 1966). At about the same time, Brodbeck and Ebner (1966) demonstrated that the enzyme involved in the biosynthesis of lactose consisted of two proteins and that α-lactalbumin was one of those proteins. Later, Hill et al (1968) showed that the other protein (β1,4-GalTase) could effect the formation of N-acetyllactosamine by itself and that α-lactalbumin inhibited this reaction. Subsequent studies showed that β1,4-GalTase was enriched in smooth membrane fractions from rat liver identifiable as Golgi stack (Coffey and Reithel 1968, Morre et al 1969). In fact, historically, β1,4-GalTase has served as a Golgi marker enzyme for cell
fractionation procedures (Farquhar and Palade 1981). Subsequent immunohistochemical localisation at the level of the EM showed the enzyme was concentrated in the trans-cisterna of the Golgi stack (Roth and Berger 1982, Rabouille et al 1995) and the trans-Golgi network (Rabouille et al 1995). β1,4-GalTase is unusual among the glycosyltransferases in that it is found to be on the cell surface as well as within the Golgi apparatus. Although this was reported over twenty years ago, the concept of glycosyltransferases being present on the cell surface remained controversial for some time. Investigations using immunohistochemical procedures and biochemical procedures have localised β1,4-GalTase to the plasma membrane of a variety of cells and tissues where it can function beyond the traditional role of glycoconjugate biosynthesis (Roseman 1970, Roth 1973, Shaper et al 1985, Shur 1991).

The general reaction catalysed by β1,4-GalTase is as follows:

\[
\text{Mn}^{2+} + \text{UDP-Gal} + \text{GlcNAc} \rightarrow \text{Gal} \beta_1 \rightarrow 4\text{GlcNAc} + \text{UDP}
\]

Although GlcNAc as a free monosaccharide is utilised as a substrate, structures with GlcNAc in β linkage to other sugar residues are much better acceptors. Thus, the apparent K_m values for GlcNAc, GlcNAcβ1→ 4GlcNAc and ovalbumin (with terminal GlcNAcβ1→ 2Man residues) are 8.3, 0.6 and 1.7 mM respectively (Beyer & Hill 1982). To date, the best structure is p-nitrophenyl-β-N-acetyl glucosamine, which has a K_m of 0.3 mM. Other β-glycosides are poor acceptors (Ebner and Magee 1975). Glucose is a good acceptor only in the presence of α-lactalbumin when lactose is formed as the end product (Schanbacher and Ebner 1970). In the absence of α-lactalbumin, β1,4-GalTase can transfer galactose to glucose to form lactose provided the glucose concentration is high (K_m = 1.4 M) (Fitzgerald et al 1970).
UDP-Gal has been shown to be the preferred sugar nucleotide donor for β1,4-GalTase. However, several studies have indicated that β1,4-GalTase can slowly and inefficiently transfer glucose, arabinose, N-acetylgalactosamine (GalNAc), and UDP-glucosamine (UDP-GICNH₂) from their UDP derivatives to GlcNAc in vitro. The rates of transfer with these alternate donors are <1% of that with UDP-Gal (Palcic and Hindsgaul 1991). Recently, it was found that α-lactalbumin induces β1,4-GalTase to utilize UDP-GalNAc at a rate 55% of that compared to the rate when UDP-Gal is the donor in the absence of α-lactalbumin (Do et al 1995). Thus, α-lactalbumin modifies both the acceptor and the donor specificity of β1,4-GalTase.

β1,4-GalTase requires Mn²⁺ for its activity. Other bivalent metal ions such as Mg are much less efficient in activating β1,4-GalTase (Babad and Hassid 1966, Navaratnam et al 1986). Kinetic studies demonstrated two types of Mn²⁺-activated sites in β1,4-GalTase; a high-affinity Mn²⁺-binding site (site 1) requiring micromolar concentrations of mangenese and a low-affinity site requiring millimolar concentrations of Mn²⁺ (site 2) (Powell and Brew 1976). It has been proposed that site 1 participates directly in the reaction mechanism, whereas site 2 is a regulator site allosterically activated by a basic protein (Navaratnam et al 1988).

β1,4-GalTase also requires a sulfhydryl group. Babad and Hassid and later other investigators showed that a number of sulphhydryl group specific inhibitors including p-chloromercuribenzoate, N-ethylmaleimide and S-mercuric-N-dansylcysteine can inactivate β1,4-GalTase (Magee and Ebner 1974, Ebner and Magee 1975, Wong and Wong 1984). Although it had been suggested that the reactive sulfhydryl group is near the UDP-Gal binding site (the inhibition is prevented by the presence of UDP-Gal but not by GlcNAc or glucose, Ebner and Magee 1975), a study using site-directed mutagenesis revealed that the cysteine at position 340 is the only cysteine residue that reacts with the suphhydryl reagents and it is also located in that part of the β1,4-GalTase peptide chain where UDP-Gal binding occurs (Wang et al
This study also showed the location of two other cysteine residues (positions 129 and 245) involved in the formation of an internal disulfide bond which has an important function in β1,4-GalTase secondary structure. Substitution of these two cysteine residues with another amino acid completely abolished the catalytic activity of β1,4-GalTase.

Two precursor polypeptides with molecular weights of 44 kDa and 47 kDa on SDS-PAGE have been identified for β1,4-GalTase at the level of rough endoplasmic reticulum (Strous 1986). After migration to the Golgi complex (20 min transit time), the apparent molecular weight of β1,4-GalTase precursors increases to 54 kDa, probably due mainly to glycosylation. β1,4-GalTase is retained in the Golgi with a half-life of ~20 hours and is also transported to the plasma membrane in some types of cells (Strous and Berger 1982, Strous 1986). The secreted β1,4-GalTase shows a molecular weight of 52 kDa (Strous 1986). Some investigators have also reported the presence of β1,4-GalTase dimers in vivo (Navaratnam et al. 1988, Fleischer et al. 1993, Yamaguchi and Fukuda 1995). The Golgi retention mechanism of β1,4-GalTase has not been fully understood yet. Sequences within and adjacent to the transmembrane domain of Golgi glycosyltransferases have been shown to be important for Golgi retention (Munro 1991, Nilsson et al. 1991, Aoki et al. 1992, Burke et al. 1992, Colley et al. 1992, Russo et al. 1992, Tang et al. 1992, Teasdale et al. 1992, Wong et al. 1992). However, the lack of amino acid sequence homology in these regions among the Golgi enzymes makes the existence of a peptide sequence for a Golgi retention signal unlikely. Some investigators have proposed that the Golgi enzymes form large oligomers via their lumenal and transmembrane domains. The oligomers then attach to the matrix via the cytoplasmic domains, thereby preventing Golgi enzymes from entering the transport vesicles (Weisz et al. 1993, Slusarewics et al. 1994, Nilsson et al. 1994, Yamaguchi and Fukuda 1995). Other investigators have suggested the length of the transmembrane domains and the lipid composition of the
membranes in the Golgi complex as important factors in Golgi retention (Pelham and Munro 1993, Masibay et al 1993).

β1,4-GalTase was the first glycosyltransferase to be cloned and sequenced. cDNA sequences have been reported for β1,4-GalTase from bovine (Shaper et al 1986a, Narimatsu et al 1986, D'Agostaro et al 1989, Masibay and Qasba 1989, Joziasse et al 1989, Russo et al 1990), murine (Shaper et al 1988, Nakazawa et al 1988, Lopez et al 1991) and human (Appert et al 1986, Masri et al 1988, Watzele and Berger 1990, Uejima et al 1992, Chattergee et al 1995) sources. The genomic structure of β1,4-GalTase has been described for the murine (Hollis et al 1989) and human (Mengle-Gaw et al 1991) gene.

Comparison of the deduced amino acid sequences of glycosyltransferases cloned to date and presumed to be Golgi-localised reveals that despite having no primary sequence homology, these enzymes share a common domain structure (Paulson and Colley 1989, Kleene and Berger 1993). It is predicted that these glycosyltransferases have a characteristic type II membrane topology consisting of a short N-terminal cytoplasmic tail, a signal/anchor transmembrane domain, an extended putative "stem" region and a large and tightly-folded globular C-terminal catalytic domain oriented within the luminal side (Figure 1.3).

The stem region, besides serving as a flexible tether to allow the catalytic domain to bind substrates of glycosylation reactions, is proposed to be accessible to proteolytic cleavage which releases the soluble form of the enzyme. Soluble forms of β1,4-GalTase, and other glycosyltransferases, have been found and purified from mammalian secretions such as colostrum and milk, and from body fluids such as blood (Beyer et al 1981, Sadler 1984). Although the soluble forms of these enzymes retain catalytic activity, whether they are of physiological relevance is still poorly understood.
The location and nature of the functional sites that are involved in binding metal ions, nucleotide sugar donors, acceptor substrates and α-lactalbumin are gradually being unravelled. Studies by Yadav and Brew (1991) provide a model for the general structure of β1,4-GalTase, in which the region between residues 93 and 250 (which contains the single disulphide bond of the β1,4-GalTase molecule) is a domain that acts in binding acceptor substrates and α-lactalbumin, while the C-terminal region (residues 275-end) is the UDP-Gal binding domain. Use of site-directed mutagenesis indicated that amino acid residues 305-308 in β1,4-GalTase are most probably involved in β1,4-GalTase catalysis or are located close to the the UDP-Gal binding region but are not involved in the binding of manganese (Zu et al/ 1995). Mutations made at residues 309, 310, and 312 were also found to affect β1,4-GalTase activity. This study also showed that the residue 309 (Tyr) is likely involved in the UDP-Gal binding. Kinetic studies of mutated β1,4-GalTase have shown that
Tyr284, Tyr309, and Trp310 are critically involved in GlcNAc binding and Tyr309 is additionally involved in UDP-Gal binding (Aoki et al. 1990). These results indicate that UDP-Gal also binds to residue(s) nearby where GlcNAc binds. Based on its functional connection with UDP-Gal binding (Navaratnam et al. 1988, Powel and Brew 1976), the weaker relatively nonspecific cation binding site can be expected to be located in the C-terminal domain. Residues 317-320, which are highly conserved and the most acidic site in the whole β1,4-GalTase molecule, can be expected to have an affinity for a variety of inorganic and organic cations. The location of the high affinity binding site for manganese whose occupation is essential for catalytic activity and is distant from the lower affinity cation site remains unclear (O’Keeffe et al. 1980, Yadav and Brew 1991).

A single gene encoding β1,4-GalTase has been localised to human chromosome 9 at band p13 and to mouse chromosome 4 (Shaper et al. 1986b, Hollis et al. 1989). Both human and murine gene loci span ~50 Kb of genomic DNA and are comprised of six exons (Hollis et al. 1989, Mengle-Gaw et al. 1991). Exon 1 encodes the 5’ untranslated region, N-terminal cytoplasmic domain, putative transmembrane region and the first 93 residues of the C-terminal domain. The remainder of the C-terminal catalytic domain (residues 94-354) is encoded by exons 2-6. The 2.9 kb 3’ untranslated region is also encoded by exon 6. The overall amino acid sequence homology for β1,4-GalTase cloned from different species (human, bovine and murine) is 80% or greater, which is quite high, with the least homology found in the stem region (Paulson and Colley 1989).

More recently, the first partial cDNA coding for a non-mammalian homolog of β1,4-GalTase was isolated from embryonic chicken brain. The predicted amino acid sequences from this cDNA, however, did not match sequences determined from tryptic peptides of chicken serum β1,4-GalTase (Kleene and Berger 1993).
β1,4-GalTase is unique among the glycosyltransferases cloned so far in that the gene locus has been shown to have two transcription initiation sites and therefore to specify two sets of mRNA transcripts encoding two forms of the protein which have a 13 amino acid difference in the length of the N-terminal cytoplasmic domain (Shaper et al 1988). The longer mRNA transcript (4.1 kb) initiates upstream of the first in-frame ATG codon and encodes a protein of 399 amino acids. The shorter, more abundant transcript (3.9 kb) initiates between the two in-frame ATG codons and encodes a protein of 386 amino acids (Harduin-Lepers et al 1993). Two forms of human β1,4-GalTase proteins with different molecular weights were first described by Strous et al (1988). The functional significance of the two forms is still under investigation. One hypothesis suggests that the two different isoforms may be involved in differential targeting of β1,4-GalTase to the Golgi and cell surface. Golgi retention signals of β1,4-GalTase has been found to lie within and adjacent to the transmembrane domain (Munro 1991, Nilsson et al 1991, Aoki et al 1992, Burke et al 1992, Colley et al 1992, Russo et al 1992, Tang et al 1992, Teasdale et al 1992, Wong et al 1992, Yamaguchi and Fukuda 1995). It is suggested that the cytoplasmic extension of the long form of β1,4-GalTase overrides the Golgi retention signal in the transmembrane domain, such that a portion of this isoform is transported to the cell surface in a cell-type specific manner (Lopez et al 1991). The exact mechanism of this differential targeting is still not clear.

The β1,4-GalTase gene appears to be expressed constitutively, at moderate levels, in most somatic tissues or cell types, a characteristic defined for the housekeeping genes. However, different levels of expression of β1,4-GalTase gene are observed in brain tissue, male germ cells and the mid- to late pregnant and lactating mammary gland (Harduin-Lepers et al 1993). This group showed that the β1,4-GalTase gene is under the control of multiple promoters with the promoter controlling the first transcription initiation site lacking in the shorter transcript. They demonstrated that in cells and tissues that express low transcript levels (such as brain tissue) the 4.1-kb
transcriptional start site is apparently used exclusively. Increased transcription from the 4.1-kb start site and low levels of transcription from the 3.9-kb start site result in the intermediate β1,4-GalTase transcript levels that are found in almost all somatic cell types. Very high transcript levels are observed in mid- to late pregnant and lactating mammary gland correlating with the predominant use of the 3.9-kb transcriptional start site. Several GC boxes were identified in the promoter region of β1,4-GalTase just upstream of the 4.1-kb transcriptional start site, which supports the housekeeping properties of the gene. The authors suggested that the region upstream of the 3.9-kb transcriptional start site, containing both positive and negative regulatory elements, functions as a mammary cell specific promoter.

Male germ cell expression of β1,4-GalTase differs from the above in that there is an additional level of transcriptional regulation. In early germ cell development, spermatogonia use only the 4.1-kb start site, but as these cells develop into spermatocytes, β1,4-GalTase gene expression is reduced to barely detectable levels. Continued differentiation to haploid round spermatids is associated with the exclusive appearance of two germ cell-specific transcripts of 2.9- and 3.1-kb which have the same open reading frame as the 4.1-kb transcript but are distinguished by the presence of an additional 5'-untranslated sequence of ~560 nucleotides. The germ cell specific promoter, located immediately upstream and contiguous with the 4.1-kb transcription start site, lacks SP1-binding sites and contains two cAMP-responsive element (CRE)-like sequences (Shaper et al 1994). Therefore, separate promoters appear to regulate housekeeping, mammary cell-specific, and germ cell-specific expression of the β1,4-GalTase gene.
1.3.2 β1,4-GalTase enzyme activity in RA

1.3.2.1 Human RA

Using ovalbumin as acceptor, Axford et al. (1987, 1992) have detected reduced enzymatic activity in B cells, and in one study in T cells, but raised activity in peripheral blood mononuclear cells from patients with RA. However, others (Furukawa et al. 1990) have reported that the defect in B cell β1,4-GalTase can be detected only when asialo-agalacto IgG is used as the acceptor and that B cell homogenates from RA patients can galactosylate asialo-agalacto-transferrin as efficiently as B cell homogenates from normal controls. Wilson et al. (1993) reported that the β1,4-GalTase enzyme is aberrantly controlled in peripheral B lymphocytes from patients with RA, however, they have detected a difference depending on the method of lymphocyte preparation. Studies by Keusch et al. (1995) detected no difference in the enzymatic activity of β1,4-GalTase in B cells from patients with RA compared to normal controls when GlcNAc-pITC-BSA was used as the acceptor substrate. Therefore, it seems that the glycoprotein acceptor and/or the method of B cell preparation used in each study contributes to the observed activity of the β1,4-GalTase enzyme.

1.3.2.2 Murine models of arthritis

In MRL lpr/lpr mice, Imai et al. (1988) reported reduced binding to lpr lymph node T cells of the lectins RCA and allo A (Allomyrina dichotoma agglutinin), which bind primarily to a Galβ1-4GlcNAc sequence. Contrary to this observation, the same authors reported an increase in the activity of β1,4-GalTase in lymph node T cells using asialo-agalacto-transferrin as the acceptor (Imai et al. 1988). Axford et al. (1994) have reported a reduction in peripheral but not splenic B lymphocyte β1,4-GalTase activity in MRL lpr/lpr
mice compared to CBA/Ca mice, and no significant difference in B cell β1,4-GalTase activity when comparing MRL lpr/lpr with MRL +/-.

1.3.3 β1,4-GalTase associated protein kinase

While attempting to isolate a β1,4-GalTase cDNA clone using a rabbit polyclonal antibody, Humphreys-Beher et al. (1986) isolated a clone which turned out to be the cDNA for a protein kinase. Molecular characterisation of the cDNA indicated that it encoded a 58 kDa serine/threonine protein kinase which had co-purified with the β1,4-GalTase preparation used for immunisation (Bunnell et al. 1990a). Sequencing of the full length cDNA revealed striking sequence homology with the cell division control protein kinases (Bunnell et al. 1990b). This protein kinase, which was called p58 galactosyltransferase-associated (p58<sup>GTA</sup>) protein kinase, has also been referred to as p58<sup>clk-1</sup> (Eipers et al. 1992) and has been designated PITSLRE-1 using the nomenclature of Meyerson and colleagues (1992). Expression of p58<sup>GTA</sup> protein kinase in CHO cells suggested that it might function to control some aspects of cell cycle regulation (Bunnell et al. 1990b). Transient expression of a p58<sup>GTA</sup> protein kinase cDNA in COS cells resulted in an approximately 3-fold increase in β1,4-GalTase activity, but to no substantial change in sialyltransferase activity (Bunnell et al. 1990a). The increase in β1,4-GalTase activity, however, did not parallel an increase in β1,4-GalTase mRNA or protein levels. Bunnell et al. (1990a) also showed that p58<sup>GTA</sup> protein kinase phosphorylated the β1,4-GalTase proteins <i>in vitro</i>, and that dephosphorylation of β1,4-GalTase proteins <i>in vitro</i> resulted in an approximately six-fold decrease in enzyme activity. Furthermore, a specific p58<sup>GTA</sup> antibody inhibited β1,4-GalTase enzyme activity <i>in vitro</i>. Thus, the authors suggest that the p58<sup>GTA</sup> protein kinase may modulate β1,4-GalTase activity by post-translational phosphorylation.

β1,4-GalTase is phosphorylated on serine residues soon after biosynthesis and it remains phosphorylated during its residency in the Golgi complex.
No phosphorylation of N-linked oligosaccharide is detected. Soluble enzyme contains very little phosphate, suggesting that the phosphate was incorporated largely into serines in the cytoplasmic or membrane-proximal stem region. In human β1,4-GalTase there are two serines in the cytoplasmic tail of long β1,4-GalTase, a single serine in the cytoplasmic tail of short β1,4-GalTase, and six serines in the stem region on the membrane-proximal side of the putative protease cleavage site involved in the generation of the soluble form of the enzyme (Masri et al 1988).

1.4 Genetic disorders affecting glycosylation

Genetic defects or polymorphisms in glycosylation are rare in intact organisms, and when they do occur have highly variable consequences. In humans, the effect of genetically altered glycosylation range from several lethal diseases, such as I-cell disease to apparently unremarkable consequences such as the ABO blood group polymorphisms. A likely possibility is that the great majority of these mutants cause lethal aberrations that prevent completion of embryogenesis. Another possibility is that mutations in glycosylation remain undetected because of alternate or “fail-safe” mechanisms that ensure that vital biological functions are carried out by more than one pathway (Varki 1993).

1.4.1 Lysosomal storage diseases

Lysosomal storage diseases are a group of inherited neurometabolic diseases, the majority of which result from the absence of one or more lysosomal enzyme (Kornfeld 1986). The genetic defect is the failure to synthesize an active form of the relevant enzyme or the inability to transport an active enzyme to the lysosome. This group of disorders constitute more than 30 different diseases amongst them are pseudo-Hurler polydystrophy and I-cell disease (mucolipidoses II and III), which result from partial or
complete deficiency of lysosomal enzyme UDP-GlcNAc GlcNAc-phosphotransferase; Gaucher disease, the most prevalent lysosomal storage disease transmitted as an autosomal recessive trait in which the loss in functional activity of glucocerebrosidase results in the storage of its lipid substrate in cells of the macrophage lineage; and diseases affecting fucosidases, sialidases, mannosidases.

1.4.2 Tn syndrome

Permanent mixed-field polyagglutinability (PMFP), also known as Tn syndrome, arises as a consequence of the loss of O-glycan specific galactosyltransferase (β1,3-GalTase) activity. Deficiency of β1,3-GalTase leads to incomplete O-glycan biosynthesis resulting in the exposure of the truncated O-glycan structure GalNACα1-O-Ser/Thr (Tn antigen) on all glycoproteins synthesised in cells affected by this enzyme deficiency. Exposed Tn antigen as a result of this deficiency is recognised by natural antibodies present in most human adult sera, leading to polyagglutinability of red cells, variable haemolytic anaemia and thrombocytopenia. Tn syndrome is a very rare acquired clonal disorder affecting several haematopoietic lineages. The majority of erythrocytes, but only 2-4% of peripheral blood lymphocytes are affected (Thurnher et al 1992). Cell surface sialic acid on Tn⁺ T cells was shown to be reduced by >50% when compared to TF⁺ (Galβ-1,3-GalNACα1-O-Ser/Thr) T cells. Thurnher and colleagues (1993) showed that in affected Tn⁺ T lymphocytes, β1,3-GalTase deficiency is due to a persistent and strong repression of an intact allele which can be induced by 5-azacytidine and sodium n-butyrate.

1.4.3 HEMPAS

Congenital dyserythropoietic anaemia type II is a very rare and genetically heterogeneous collection of deficiencies in various steps of N-glycosylation. This disease, which is inherited in an autosomal recessive manner, is also known as HEMPAS (hereditary erythroblastic multinuclearity associated with
the positive acidified serum lysis test) and is characterised by the lack of normal polylactosamine containing glycans in the erythrocyte membrane glycoprotein band 3 and band 4.5 and their replacement by unusual truncated hybrid structures. Red cells expressing such structures aggregate and are removed from the circulation (Fukuda et al. 1990). Some patients with HEMPAS show a deficiency of N-acetylgalactosaminytransferase II activity, while others demonstrate a deficiency of Golgi α-mannosidase II due to a defect in the 5' promoter region of the gene resulting in <10% of normal levels of the enzyme mRNA in B-cells (Fukuda et al. 1990). In another variant of congenital dyserythropoietic anaemia type II, defective glycosylation of erythrocyte membrane glycoconjugates is associated with reduced levels of membrane-bound form of β1,4-GalTase. This deficiency results in the absence of polylactosamines and the presence of high-mannose type oligosaccharides on erythrocyte N-glycans, features not detected in normal cells or in other HEMPAS cases. Due to high levels of soluble β1,4-GalTase in serum, it is thought that the mutation might have occurred in the membrane-binding domain, changing the uncleavable signal peptide into a sequence susceptible to signal peptidase, or that the mutation might result in increased susceptibility of β1,4-GalTase to other types of proteolytic cleavage (Fukuda et al. 1989).

### 1.4.4 Carbohydrate-deficient glycoprotein syndromes

The carbohydrate-deficient glycoprotein syndromes are a new class of genetic, multisystemic diseases with major nervous system involvement. They are characterised by a deficiency in the carbohydrate moiety of secretory glycoproteins, lysosomal enzymes, and probably membranous glycoproteins. The defect appears to be due to a deficiency of asparagine N-linked oligosaccharide transfer in type I disease and a deficiency of N-acetylgalactosaminytransferase II in type II disease (Jaeken et al. 1993). A recent discovery of a third variant of the disease (type III) awaits the discovery of the basic biochemical defect (Ramaekers et al. 1991).
1.4.5 Tumours

Changes in the glycosylation of cell surface molecules are associated with malignant transformation (Hakamori 1984, Singhal and Hakamori 1990). These glycosylation changes can be detected by lectins or antibodies and are used as markers of the disease which have been correlated with the tumour grade, metastatic potential and prognosis (Muramatsu 1993). Abnormal glycosylation in transformed cells are believed to be the due to changes in the concentration or activity of the processing enzymes, the glycosyltransferases and the glycosidases. For example, increased levels of GlcNAc-transferase V in many transformed cells results in an increase in β1-6 branching and polylactosaminoglycan expression (Yamashita et al 1985, Dennis et al 1987, Fernandes et al 1991). The polylactosamine sequences probably contribute to the metastatic potential by reducing binding adhesion to the extracellular matrix proteins (laminin, fibronectin and collagen) and thereby facilitating tumour cell displacement and invasion. Other oligosaccharides aberrantly expressed in cancers include the blood group antigens. A loss of expression of the Lewis antigens (Le\textsuperscript{a} and Le\textsuperscript{b}) has been observed in 50% of patients suffering from various cancers, such as of the pancreas, colon or urinary bladder (Langklide et al 1991). Tumours which have lost expression of the Le\textsuperscript{a} antigen are associated with more aggressive behaviour of cancers of the bladder, uterus, cervix, colon, head and neck. The appearance of an abnormal blood group antigen, or the expression of a normal blood group antigen in a tumour of a tissue that does not normally express that antigen, has also been detected in various tumours (Singhal and Hakamori 1990). It appears that the body’s surveillance system for monitoring tumours may be based on detecting changes in glycosylation status (Rademacher 1992). Glycosylation processing inhibitors are also being developed as potential anti-cancer agents (Dennis 1992).
1.4.6 Immunodeficiency

A major class of receptors involved in intercellular recognition during leukocyte migration and recruitment into various sites of inflammation is the selectin family of receptors. The minimal carbohydrate ligands involved in interaction with the selectins appear to be sialylated fucosylated sugar chains, such as Sialyl Lewis\textsuperscript{x} and Sialyl Lewis\textsuperscript{a}. In leukocyte adhesion deficiency type II, low fucose on many glycoconjugates results in a lack of ligands for selectins, and therefore poor leukocyte adhesion. The specific biochemical defect of this autosomal recessive disease has not yet been established, but it is thought to be a reflection of a general deficiency in fucosyltransferase enzyme. Neutrophils of the affected patients, who suffer from severe and recurrent infections, show a deficiency in Sialyl Lewis\textsuperscript{x} (Etzioni et al 1992).

The Wiskott-Aldrich syndrome is an X-linked immunodeficiency affecting T cells, B cells and platelets. Patients suffer from recurrent infections, thrombocytopenia and eczema. Lymphocytes and platelets from patients with Wiskott-Aldrich syndrome show aberrant O-glycan synthesis (Higgins et al 1991). This is probably due to increased β-1,6-GlcNAc-transferase activity which results in altered branching of O-linked oligosaccharides on CD43 molecule (leukosialin, sialophorin) and other sialoglycoproteins of lymphocytes and platelets.

1.4.7 Thyroid autoimmunity

All humans have a natural autoantibody (up to 1% of circulating IgG) against Galα1-3Galβ1-4GlcNAc sequences. The biosynthetic basis for the evolutionary suppression of the α-galactosyl epitope expression was found to be the specific suppression of the enzyme α-1,3-galactosyltransferase probably due to regulatory mutations affecting the transcription of this gene. Such aberrant expression was reported to occur in human mammary carcinoma cells (Galili 1993). It is also hypothesised that aberrant expression of α-galactosyl epitopes on human cells may result in autoimmune reactions.
(Galili 1989). Anti α-galactosyl titres were found to be elevated in patients with Graves' disease. Winand et al (1994) were able to show that these natural antibodies induce iodine uptake in thyrocytes of patients with Graves' disease, thereby resulting in an additional stimulatory effect on thyroid above that produced by autoantibodies to thyroid-stimulating hormone (TSH) receptor.
CHAPTER 2

MATERIALS AND METHODS
2.1 Cloning in plasmid vectors

2.1.1 Dephosphorylation of plasmid DNA

Plasmid DNA (10-20 µg pBluescript; Stratagene, La Jolla, Ca) was linearised by complete digestion with the desired restriction enzyme. The sample was then extracted with phenol-chloroform extraction buffer (phenol-chloroform-isoamyl alcohol [25:24:1] containing 800 µg/ml hydroxyquinolone and equilibrated with 10 mM Tris pH 8.0) and the DNA was precipitated with two volumes of ethanol for 15 minutes at 0°C. The DNA was recovered by centrifugation at 12,000g for 10 minutes at 4°C and redissolved in 90 µl of 10 mM Tris-HCl pH 8.3. A 200 ng aliquot of DNA was removed and to the remainder was added 10 µl of 10X dephosphorylation buffer (Boehringer Mannheim, Germany or Boehringer Mannheim, Indianapolis, IN; containing 10 mM ZnCl₂, 10 mM MgCl₂ and 100 mM Tris-HCl pH 8.3) and the appropriate amount of Calf intestinal alkaline phosphatase (CIP) and incubated under the appropriate conditions. The amount of CIP required for protruding 5' termini is 1 unit per 100 pmoles of 5'-terminal phosphate residues, with incubation at 37°C for 30 minutes and the amount of CIP required for blunt or recessed termini is 1 unit per 2 pmoles with incubation at 37°C for 15 minutes and another aliquot of CIP and continued incubation at 55°C for a further 45 minutes. The phosphatase was then inactivated by heating at 75°C for 10 minutes in the presence of 5 mM EDTA, pH 8.0. The dephosphorylated DNA was then extracted with phenol-chloroform buffer and precipitated by adding 0.1 volume of 3 M sodium acetate, pH 7.0 and two volumes of ethanol. The DNA pellet was washed in 70% ethanol at 4°C and redissolved in TE (10 mM Tris, 1 mM EDTA, pH 7.6) buffer at a concentration of 100 µg/ml.
2.1.2 Ligation and transformation

200 ng of dephosphorylated plasmid DNA was added to 200 ng of the foreign DNA fragment carrying compatible phosphorylated termini. The ligation reaction was set up with final concentrations of 0.05 M Tris-HCl (pH 7.6), 10 mM MgCl₂, 10 mM dithiothreitol (DTT), 50 μg/ml bovine serum albumin (Fraction V), 1 mM ATP and 1.5 units of bacteriophage T4 DNA ligase. The reaction mix was incubated at 16°C overnight. Two control test ligations were also set up to assay the efficiency of dephosphorylation and ligation: One containing 200 ng of dephosphorylated plasmid DNA and no foreign DNA and the other containing 200 ng of linearised plasmid DNA that has not been treated with CIP. Competent \textit{E. coli} cells were transformed with 1 μl of neat, 1:10 and 1:100 dilutions of each of the ligation reactions. A negative control with \textit{E. coli} cells only and a positive control with 10 pg of closed circular pBluescript vector were also set up. Transformation reactions were incubated on ice for 30 minutes, heat-shocked at 42°C for 90 seconds and chilled on ice for 3 minutes. To these mixtures were then added 1.0 ml of LB medium (Lenox broth; 10 g/l tryptone, 5 g/l yeast extract and 5 g/l NaCl, pH 7.0) and incubated at 37°C for 45 minutes with occasional shaking. To isolate single colonies, 1, 10 and 50 μl of each of the transformation reactions were gently spread (using a glass spreader with circular motion) over the surface of ampicillin LB plates (15 g/l bactoagar and 100 μg/ml ampicillin in LB medium) and incubated at 37°C overnight. Identification of bacterial colonies that contained recombinant plasmids was sometimes aided by using X-gal plates. Fresh ampicillin/LB plates were spread with 40 μl of 0.1 M IPTG (isopropylthio-β-D-galactoside) and 40 μl of 4% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) solution in dimethylformamide. Bacteria carrying recombinant plasmids produced white colonies which were distinguished from non-ligated blue colonies.
2.1.3 Screening colonies by hybridisation

Single colonies were grid on two plates: a master plate (ampicillin/LB plate) and another plate with a sterile circular Hybond-N nylon membrane (Amersham International, Amersham, UK) placed on top of the agar. Both plates were incubated at 37°C overnight. The membrane was lifted and placed on layers of filter paper (Whatman 3 MM chromatography paper) soaked in a tray containing a shallow depth of 1.5 M NaCl, 0.5 M NaOH for 4 minutes to denature bacterial proteins, followed by 4 minutes in 1.5 M NaCl, 0.5 M Tris-HCl (pH 8.0) to neutralise. The membrane was then washed in 1X SSPE (20X SSPE contains 3.6 M NaCl, 0.2 M NaH$_2$PO$_4$, 0.02 M EDTA pH 7.7) for 2 minutes on a shaker and transferred onto filter papers soaked in 3X SSC (20X SSC contains 3 M NaCl, 0.3 M sodium citrate, pH 6.15), 0.1% SDS and scrubbed with tissues soaked in the same buffer to remove protein debris. Finally, the membrane was washed in 3X SSC for 2 minutes, air dried and the DNA was crosslinked using a UV Stratalinker (Stratagene, La Jolla, Ca). Hybridisation of the membrane with the desired probe was carried out as for Southern blots. Positive colonies were picked from the master plate and the structure of the plasmids verified by restriction analysis of minipreparations of plasmid DNA.

2.1.4 Preparation of competent cells

A single *E. coli* strain colony was inoculated into 5 ml of LB medium containing 1X MMK (10 mM MgSO$_4$, 10 mM MgCl$_2$ and 2.5 mM KCl) solution and incubated at 37°C overnight. 0.5 ml of the culture was transferred to 100 ml prewarmed LB medium containing 1X MMK and grown at 37°C, shaking, to an optical densities (OD)$_{600}$ of 0.4. The culture was split into two 50-ml tubes and left on ice for 10 minutes. All the subsequent steps were performed at 4°C or on ice. The cells were harvested by centrifugation for 15 minutes at 2000g, 4°C. The supernatant was removed completely and cells were pooled and resuspended in 33 ml of an ice-cold buffer containing 10 mM CaCl$_2$, 100 mM RbCl$_2$, 50 mM MnCl$_2$, 10 mM MES (2[N-morpholino]ethane-sulphonic
acid), pH 5.8 and incubated for 30 minutes on ice. The cells were again harvested by centrifugation for 15 minutes at 4000g, 4°C, resuspended in 8 ml of another ice-cold buffer containing 10 mM PIPES (piperazine-\(\text{-N,}\text{N'\text{-bis}[2-ethane]sulphonic acid}\)), pH 6.5, 75 mM CaCl\(_2\), 10 mM RbCl\(_2\), 15% (V/V) glycerol and incubated on ice for 15 minutes. Finally, the cells were aliquoted into pre-chilled eppendorf tubes (200 \(\mu\)l/tube) and immediately stored at -70°C. To assess the competence of the cells, 25 ng of a closed circular plasmid (pBluescript) was added to 200 \(\mu\)l of competent cells and transformation was carried out using the heat-shock method described above. Aliquots of the transformed culture were plated on LB/ampicillin plates and incubated at 37°C overnight.

2.1.5 Growth in liquid media

Small freshly saturated cultures of \(E.\) \textit{coli} were prepared by inoculating 5 ml of sterile LB medium (with 100 \(\mu\)g/ml ampicillin when required) with a single bacterial colony or 50 \(\mu\)l of a frozen glycerol stock and incubating at 37°C overnight with agitation. Larger cultures in baffle flasks were inoculated with aliquots from small overnight cultures and incubated overnight at 37°C with agitation.

2.1.6 Minipreps of plasmid DNA

Isolation of small quantities of plasmid DNA from bacterial cells (i.e. minipreps of plasmid DNA) was performed according to the alkaline lysis procedure. Small cultures of bacteria were spun at 2000g for 15 minutes at 4°C. The supernatant was removed and the pellet was resuspended in 200 \(\mu\)l of glucose mix solution (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0). Then, 400 \(\mu\)l of a freshly made solution of 1% SDS, 0.2 M NaOH was added, mixed by inversion, and left on ice for 5 minutes. This was followed by the addition of 200 \(\mu\)l potassium acetate solution (5 M potassium acetate, glacial acetic acid to pH 4.8) and vigorous shaking of the tube. The lysed
solution was then transferred to an eppendorf tube and centrifuged for 10 minutes at 12,000g at room temperature to remove cell debris and chromosomal DNA. The aqueous phase containing plasmid DNA was precipitated by adding 0.6 volume isopropanol, washed in 70% ethanol and dissolved in 200 µl of TNE (10 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 8.0) buffer. Contaminating RNA was removed by incubating the sample at 37°C for 30 minutes in the presence of 20 µg/ml of RNase. Finally, the sample was extracted with phenol-chloroform extraction buffer, DNA was concentrated by adding two volumes of ethanol and washed in 70% ethanol before being resuspended in 30 µl of 10 mM Tris-HCl, pH 8.0.

2.1.7 Maxipreps of plasmid DNA
Large amounts of high-quality plasmid DNA (i.e. maxipreps of plasmid DNA) was obtained using the alkaline lysis method and centrifugation in CsCl/ethidium bromide density gradients. E. coli cells (1 litre culture) were collected by centrifugation for 30 minutes at 4°C, 2000g. The precipitated cells were resuspended in 100 ml of glucose mix solution. 200 ml of a freshly prepared solution of 0.2M NaOH/1% SDS was added followed by 100 ml of potassium acetate solution (as above). The suspension was shaken vigorously and spun for 25 minutes at room temperature, 4000g. The supernatant was strained through three layers of sterile cotton gauze and the plasmid DNA was precipitated by adding 0.6 volume isopropanol and spinning for 25 minutes at 4000g, room temperature. The pellet was washed very gently with 70% ethanol, air dried and resuspended in 5 ml of TE buffer. The solution was made up to 9 g with the same buffer, 10 g of CsCl and 1 ml ethidium bromide solution (5 mg/ml) added, sealed in a Quik-Seal ultracentrifuge tube and centrifuged in a Beckman L7 ultracentrifuge, at 55,000 rpm using a 70Ti rotor, at 20°C overnight. The plasmid band was recovered by using a 20-G needle attached to a syringe. Plasmid DNA/ethidium bromide solution was then diluted with two volumes of TE, the DNA was ethanol precipitated and centrifuged for 10 minutes at 10,000g. The
pellet was resuspended in 0.5 ml TNE buffer, RNase was added to 20 μg/ml and incubated for 30 minutes at 37°C. The solution was extracted with phenol-chloroform extraction buffer before concentrating the plasmid DNA with two volumes of ethanol. The DNA pellet was dissolved in 10 mM Tris, pH 8.0.

2.1.8 DNA purification using low melting temperature agarose gel

The insert was cut out of the plasmid vector using the appropriate restriction enzyme(s) and run alongside a marker on a 1% low melting temperature agarose gel in 1X TBE (90 mM Tris, 90 mM boric acid, 2.5 mM EDTA) solution containing 0.5 μg/ml ethidium bromide. The insert band was cut out, and the agarose gel melted by heating at 65°C for 5 minutes. The sample was then extracted with phenol containing 800 μg/ml hydroxyquinolone (equilibrated with 0.3 M sodium acetate, pH 4.9) for at least three times until no white interface was visible. Sodium acetate was then added to a final concentration of 0.2 M and the solution was extracted using standard phenol-chloroform extraction buffer. The DNA was precipitated with two volumes of ethanol and left for 30 minutes on dry ice (or at -70°C overnight). After centrifugation at 12,000g for one hour at -20°C, the pellet was resuspended in 10 mM Tris, pH 8.0. To estimate the amount of DNA recovered, 1 μl was run on an agarose gel together with a known amount of a marker.

2.2 Isolation and analysis of DNA

2.2.1 Isolation of genomic DNA

2.2.1.1 Source of human DNA

Heparinised peripheral blood was treated with ammonium chloride lysis buffer (155 mM NH₄Cl, 10 mM NH₄HCO₃, 0.1mM EDTA, pH 7.4) for 15 minutes at 4°C to lyse erythrocytes. The leukocytes were washed twice in Hank's
Balanced Salt Solution (HBSS) and then resuspended at $2 \times 10^5 - 2 \times 10^6$ cells/ml in TNE buffer.

2.2.1.2 Source of murine DNA

Freshly-isolated liver was homogenised in TNE buffer using a glass homogeniser and warmed for a few minutes at 37°C before removal of proteins.

2.2.1.3 Extraction of DNA

Proteinase K (Boehringer) was added to pre-warmed cell suspensions (or liver homogenate) to a final concentration of 100 µg/ml in the presence of 1% SDS to promote dissociation of proteins from nucleic acids. Digestion was carried out at 37°C for 2 hours or overnight. The sample was handled very gently from this moment on to reduce damage by mechanical shearing. Proteins were removed from the solution by adding an equal volume of phenol-chloroform extraction buffer and mixing very gently by inversion until an emulsion formed. After centrifugation at 4000g for 15 minutes at room temperature, the aqueous phase was withdrawn with a wide-mouthed bent glass pipet. DNA was then precipitated by adding 2.5 volumes of absolute ethanol and swirling very gently until a stringy DNA precipitate was formed which was then fished out using a sealed pasteur pipet and washed in 70% ethanol and dried before being transferred to 5-10 ml of TNE buffer. Dissolution of DNA in TNE buffer was aided by gentle rolling at 37°C for 2 hours and leaving at 4°C overnight. The DNA solution was then warmed to 37°C and contaminating RNA removed by adding heat-treated RNase (treated at 95°C for 30 minutes) to a final concentration of 20 µg/ml and incubated at 37°C for 30 minutes. A second round of purification was then carried out by repeating the proteinase K/SDS treatment, phenol extraction and ethanol precipitation steps. The DNA precipitate was finally dissolved in 10 mM Tris, pH 8.0. The amount of DNA was quantified by measuring the
absorbance at 260 nm of the solution in a cuvette with a 1-cm path length and calculated as follows:

\[ \text{DNA (\(\mu g\))} = \text{Absorbance (260 nm) \times 50 \(\mu g/ml\)} \]

The ratio of the absorbance at 260 to 280 nm was used as an indication of purity. Values for DNA solutions of 1.8 to 1.9 are acceptable.

### 2.2.2 Southern blotting assays

#### 2.2.2.1 Restriction enzyme digestion

Ten micrograms of genomic DNA was digested to completion with 25-50 units of restriction endonuclease according to the manufacturer's instructions. (Amersham, Boehringer, New England Biolabs, Hertfordshire, UK and Pharmacia Biotechnology, Buckinghamshire, UK). Concomitantly, 3 \(\mu l\) of the reaction mixture was mixed with 0.5 \(\mu g\) of phage \(\lambda\) DNA and incubated under the same conditions. The efficiency of digestion was checked by analysing the \(\lambda\) DNA sample on a 0.7% agarose gel. After complete digestion, the genomic DNA sample was phenol extracted and the restriction fragments were separated on a gel.

#### 2.2.2.2 Agarose gel electrophoresis

Restriction fragments were separated by electrophoresis in 0.6% agarose gels. Horizontal agarose slab gels consisted of 0.6% agarose in TBE (90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, pH 8.0) buffer containing 0.5 \(\mu g/ml\) ethidium bromide. The samples were mixed with loading buffer (20% ficoll in 10 mM Tris, 1 mM EDTA, pH 8.0 containing Orange G) and run into the gel at 20 volts and then separated at 50 volts until the dye front had run the required length. Standard markers of phage \(\lambda\) DNA cut with HindIII were run on every gel (fragment sizes: 23.1, 9.4, 6.5, 4.3, 2.3, 2.0 and 0.6 kb). DNA bands were visualised using a UV trans-illuminator and photographed with a Polaroid land camera.
2.2.2.3 Transfer of DNA onto nylon membrane

Prior to blotting, the gel was treated with 0.125 N HCl for 20 minutes for partial hydrolysis, followed by denaturation in 0.5 N NaOH, 1.5 M NaCl for 40 minutes and neutralisation in 1.5 M NaCl, 0.5 M Tris pH 8.0 for 40 minutes. The fragments were then capillary-transferred from the gel to a Hybond-N nylon membrane using 20X SSC (3 M NaCl, 300 mM sodium citrate, pH 6.15) and the DNA was crosslinked to the membrane using a UV Stratalinker.

2.2.2.4 Hybridisation and washing the membrane

Blots were prehybridised for a minimum of two hours at 65°C in hybridisation buffer (450 mM sodium chloride, 45 mM sodium citrate pH 6.15, 0.2% ficoll, 0.2% BSA fraction V, 0.2% polyvinylpyrrolidone, 0.1% SDS, 10% dextran sulphate, and 50 μg/ml of denatured salmon sperm DNA). Stock salmon sperm DNA was prepared by boiling at a concentration of 10 mg/ml in 0.2 M NaOH for 30 minutes, followed by addition of Tris to 100 mM, neutralisation to pH 7.0 with HCl, addition of NaCl to 0.4 M, phenol extraction three times, ethanol precipitation and then dissolving in 10 mM Tris. Hybridisation with $^{32}$P-labelled cDNA probes (see section 2.2.4) for murine β1,4-GalTase, murine P58$^{GTA}$ or human β1,4-GalTase was carried out in the above solution at 65°C in a shaking waterbath overnight. The membranes were then given three 15 minute washes in 3X SSC, 0.1% SDS, followed by three further washes in 0.3X SSC, 0.1% SDS. All the washes were carried out at 65°C. After a rinse in 2X SSC the membranes were dried and exposed to Kodak X-AR5 film at -70°C using a screened autoradiography cassette. The probes were removed by washing the membranes in 0.2 M Tris pH 7.5, 0.1% SDS and 0.1X SSC for 30 minutes at 45°C. The removal of the probe was checked by exposing the membrane to X-ray film before hybridising with the next probe.
2.2.3 Isolation of cDNA probes

2.2.3.1 Murine β1,4-GalTase
The murine β1,4-GalTase cDNA probe (Pouncey et al. 1990) was approximately 2.3-kb in size and contained 1.6-kb of murine β1,4-GalTase cDNA, corresponding to nucleotides 241-1884 of the sequence published by Shaper et al. (1988). The cDNA probe, provided by Dr V. J. Kidd as plasmid DNA in ethanol, was a BamHI-HindIII insert in pBR322. Competent E. coli (DH5α) bacteria (prepared as in section 2.1.1.4) were transformed with 25 ng of plasmid DNA, grown on LB-ampicillin plates and minipreps analysed for selected colonies. Large amounts of recombinant plasmid DNA were obtained by performing a maxi-prep on one of the positive colonies and the plasmid DNA digested with BamHI and HindIII to release the 2.3-kb insert. The probe was finally purified by running the digested sample on a low-melting point agarose gel.

2.2.3.2 Murine p58GTA
The murine p58GTA cDNA probe was 1.9-kb in size and contained a 478-bp 5' untranslated region, an open reading frame encoding a 439-amino acid sequence and a 152-bp 3' untranslated region (Bunnell et al. 1990). The murine p58GTA, provided by Dr V. J. Kidd as a glycerol stock, was full length and inserted in the XhoI site of pKS+. Competent cells (E. coliDH5α) were transformed, a maxiprep made and the probe purified as above using XhoI to release the 1.9-kb insert.

2.2.3.3 Human β1,4-GalTase
The human β1,4-GalTase DNA probe (Mengle-Gaw et al. 1991) was a mixture of five genomic fragments corresponding to pgt1 (350 bp SacI), pgt2 (1.7 kb SacI-EcoRI), pgt3 (800 bp HincII), pgt4 (750 bp EcoRI-StuI) and pgt6i (1.9 kb HindIII) as shown in Figure 3.1. These probes were provided by Dr L.
Mengle-Gaw as ethanol precipitate of both genomic DNA fragments and inserts in plasmid pUC18.

2.2.4 Synthesis of radiolabelled DNA probes
Uniformly labelled DNA probes were synthesised using random oligonucleotide primers (Amersham multiprime™ kit). The reaction mixture was set up with 25 to 100 ng of DNA probe (boiled at 95°C for 5 minutes), 10 μl of a mixture of dATP, dTTP, dGTP in a concentrated multiprime DNA labelling buffer, 5 μl of random hexanucleotide primers, 3 μl $^{32}$P-α-dCTP (800 Ci/mmol, 10 mCi/ml), 2 μl of “Klenow” DNA polymerase and distilled water to 30 μl total volume. This solution was incubated at 37°C for 30 minutes. Before adding the DNA probe to the hybridisation solution, unincorporated nucleotides were separated from radiolabelled DNA probe using a NucTrap™ push column (Stratagene) according to the manufacturer's instructions. Briefly, the column was pre-wet by adding 70 μl of 1X STE (100 mM NaCl, 20 mM Tris-HCl pH 7.5, 10 mM EDTA). The sample was then loaded in a volume of 70 μl and pushed through the column resin. Finally, the column was washed by adding another 70 μl aliquot of 1X STE.

2.3 Isolation and analysis of RNA

2.3.1 Isolation of RNA

2.3.1.1 Source of human RNA
a) Separation of human peripheral blood mononuclear cells
Human peripheral blood was diluted 1:1 with medium (RPMI 1640; Life Technologies, Renfrewshire, UK) and layered carefully over Lymphoprep (Nycomed, Oslo, Norway) with a 2:1 ratio and mononuclear cells (PBMCs) were isolated by centrifugation at 800g for 20 min at 18°C. Cells were removed from the interface and washed twice in HBSS.
b) Separation of human peripheral blood lymphocytes

To isolate peripheral blood lymphocytes, PBMCs obtained as above were resuspended at 5 × 10^6/ml in pre-warmed (37°C) RPMI 1640 medium containing 20% fetal calf serum (FCS), 2 mM L-glutamine and 50 units/ml penicillin-streptomycin. Monocytes were depleted by plastic adherence (Gibco tissue culture flasks) at 37°C for one hour, and the non-adherent cells (lymphocytes) washed twice in HBSS prior to use (all tissue culture reagents and plastics were supplied by Life Technologies).

c) Separation of peripheral blood B-lymphocytes

Purified B-cells were obtained by positive selection from PBMCs using anti-CD19 coated Dynabeads M-450 (Dynal, Oslo, Norway) following the manufacturer's instructions. Briefly, Dynabeads M-450 Pan-B (CD19) were washed three times in PBS/FCS (0.15 M NaCl in 0.01 M Sodium phosphate pH 7.4 containing 2% FCS) and resuspended in an equal volume of PBS/FCS to the original starting volume. The Dynabeads were then added to ice-cooled PBMCs at a ratio of 3:1 (bead : cell) and incubated at 4°C for 15 minutes with gentle tilting and rotation. The rosetted CD19^+ B-cells were isolated by placing the sample in a magnetic particle concentrator (Dynal MPC) for 2 minutes. The supernatant was poured off and the rosetted cells were gently resuspended in PBS/FCS, taking care not to disrupt the rosettes. The rosetted cells were washed five times in PBS/FCS as above before RNA isolation. This population comprised >95% CD19^+ cells as estimated by examining an aliquot of the final suspension under a UV microscope for the proportion of rosetted and free cells.

d) B-cell enrichment

i. Preparation of neuraminidase-treated sheep erythrocytes

Sheep red blood cells (SRBC) were washed four times by adding 30 ml RPMI 1640 to 20 ml SRBC and spinning at 1000g for 10 minutes. One ml of
neuraminidase solution (3 mg/ml in RPMI filtered through a 0.22 µm millipore filter) was added to one ml of washed SRBC pellet, the cells were resuspended gently and incubated for one hour at 37°C with intermittent inversion of the tubes. The SRBCs were then washed four times in medium, resuspended in 50 mls of complete medium (RPMI 1640, 2 mM L-glutamine, 50 units/ml penicillin-streptomycin and 10% FCS), and stored at 4°C for up to two weeks, or until evidence of haemolysis was apparent, whichever period was the shorter.

ii. Sheep red blood cell rosetting of PBMCs

Peripheral blood lymphocytes (5x10^7 cells in 5 ml complete medium) were mixed with 5 ml of neuraminidase-treated SRBC and 5 ml FCS and incubated in a 37°C waterbath for 10 minutes and then left in an ice-bath overnight. The supernatant was removed and the cell pellet was gently resuspended by rolling the tube. The cell suspension was then layered on Lymphoprep and centrifuged for 15 minutes at 800g, 4°C. The interface of B-enriched (E-negative) cells was collected, washed twice and resuspended in complete medium.

2.3.1.2 Source of murine RNA

a) Preparation of spleen cell suspension

Spleens were removed into ice-cold medium (RPMI/5% FCS), passed through a 20 µm wire mesh (in a petri dish on ice) and allowed to settle for 1-2 minutes. The separated cells were removed and washed in medium. Cells were centrifuged at 350g for 5 minutes and the pellet gently resuspended into medium. After centrifugation, erythrocytes were removed by resuspending the pellet in 5 ml of lysing buffer (0.16 M ammonium chloride pH 7.2) for 5 minutes. The cell suspension was topped up with medium and centrifuged as above. The cells were washed twice in medium before use.
b) Preparation of splenic lymphocytes

To remove macrophages, the spleen cells were resuspended (10^7 cells/ml) in pre-warmed RPMI 1640 medium containing 20% FCS and incubated at 37°C on a large surface area for one hour. The non-adherent cells (lymphocytes) were removed and washed twice in RPMI 1640 medium before use.

c) Preparation of lymph node cell suspension

Lymph nodes were removed into ice-cold medium containing 5% FCS. Cell suspensions were prepared (as for spleen above) from pools of lymph nodes.

d) Preparation of surface IgG^+ and surface IgG^- cells

The B-cells from the mouse spleen and lymph node cell suspensions were isolated using magnetic beads coated with anti-mouse IgG antibodies (Dynal), as described in section 2.3.1.1c, with PBS/FCS (0.15 M PBS pH 7.4 containing 2% FCS) as the buffer. This positively selected population consisted of >95% surface IgG^+ (slgG^+) cells. The surface-IgG negative (slgG^-) population was obtained by two more rounds of depletion using anti-mouse IgG coated Dynabeads. These cells were then placed on plastic at 37°C for 1 hour to remove adherent cells, following which the non-adherent cells were washed twice in PBS prior to RNA isolation. This cell population consisted of >70% CD3^+, <10% Mac-1^+ and <1% slgG^+ in the case of spleen, and >85% CD3^+, <2% Mac-1^+, and <1% slgG^+ cells in the case of lymph node.

2.3.1.3 Counting of cells

Cell viability was determined by staining with acridine orange and ethidium bromide and scoring under ultraviolet microscopy to distinguish viable (green) and non-viable (orange) cells. Cell counts were performed using a haemacytometer (an improved Neubauer chamber, at least 200 cells were counted) and ethidium bromide/acridine orange stain (equal volumes of
0.001% acridine orange in PBS and 0.02% ethidium bromide in PBS were mixed and diluted 1:10 before use).

2.3.1.4 Characterisation of cell populations

Various monoclonal antibodies against specific cell-surface markers were used in direct immunofluorescence to evaluate the different cell populations. Cells (1-5x10^6/ml) were washed twice with PBS/1% BSA and centrifuged for 10 minutes at 450g (350g for murine cells), 4°C. After removing the supernatant, the pelleted cells were resuspended in the remaining buffer and incubated with the labelled monoclonal antibody for 40 minutes on ice under saturating conditions (according to manufacturer's instructions). After three washes, the cells were resuspended in 200 μl PBS/1% paraformaldehyde and analysed by standard flow cytometry on a FACScan (Becton Dickinson, Mountain View, Ca). Data were collected for 5-10,000 cells on FACS/Consort 30 with linear amplification of forward and side-scatter and logarithmic amplification of fluorescence emission. Dead cells and cellular debris were gated out on the basis of forward and side-scatter. The percentage positively staining cells was determined from cutoffs set based on isotype-controls. Calculations were carried out using FACScan software. Monoclonal antibodies and isotype controls for the specificity of immunostaining, conjugated to either fluorescein isothiocyanate (FITC) or R-phycoerythrin (RPE) used in the analyses included:

FITC-conjugated monoclonal rat anti-mouse T cell, CD3 (SeroTec, Oxford, UK)
FITC-conjugated goat anti-mouse IgG (whole molecule) (Sigma, Poole, UK)
FITC-conjugated goat anti-mouse polyvalent immunoglobulins (IgG, IgA, IgM) (Sigma)
RPE-conjugated monoclonal rat anti-mouse macrophage, MAC-1 (CD11b, SeroTec)
controls: FITC-conjugated rat IgG2a (CALTAG Laboratories Inc. San Francisco, Ca)
    FITC-conjugated goat IgG (Sigma)
    RPE-conjugated rat IgG2b (CALTAG)
2.3.1.5 Extraction of RNA

Total RNA was isolated using the single-step method of acid guanidium thiocyanate-phenol-chloroform extraction (Chomczynski et al. 1986). Tissues (or cells) were homogenised in a denaturing solution containing 4 M guanidium thiocyanate/25 mM sodium citrate, pH 7.0/0.5% sarcosyl/0.1 M 2-mercaptoethanol (1 ml of denaturing solution per $10^7$ cells or 100 mg of tissue). The homogenate was mixed sequentially with 1/10 volume 2 M sodium acetate, pH 4.0, equal volume of water-saturated phenol and 1/5 volume chloroform-isooamyl alcohol mixture (49:1). The homogenate was mixed thoroughly by inversion after the addition of each reagent. The final suspension was shaken vigorously for 10 seconds and cooled on ice for 15 minutes. The sample was then centrifuged at 10,000 g for 20 minutes at 4°C. The upper aqueous phase was transferred to a fresh tube, mixed with one volume isopropanol and then placed at -20°C overnight. The total RNA was pelleted by centrifugation for 20 minutes at 10,000 g, 4°C. The RNA pellet was redissolved in denaturing solution, reprecipitated with isopropanol, washed in 70% ethanol and finally resuspended in DEPC-treated water (double-distilled water [ddH$_2$O] treated with 0.1% diethylpyrocarbonate [DEPC], shaken vigorously, left at room temperature overnight and autoclaved before use), containing 0.1 mM EDTA by heating at 65°C for 10 minutes. Samples were quantitated and stored at -70°C. The amount of RNA was quantified by measuring the absorbance at 260 nm of the solution in a cuvette with a 1-cm path length and calculated as follows:

\[
\text{RNA (\mu g)} = \text{Absorbance (260 nm)} \times 40 \mu g/ml
\]

The ratio of the absorbance at 260 to 280 nm was used as an indication of purity. Values for RNA solutions of 1.9 to 2.0 are acceptable.
2.3.2 Northern blotting assay

2.3.2.1 Preparation of agarose/formaldehyde gel
The electrophoresis tank, the gel casting tray, pump tubings and the comb were cleaned with detergent, rinsed with distilled water and dried with ethanol. All the equipment was then soaked in a solution of 3% hydrogen peroxide for 10 minutes at room temperature and then rinsed thoroughly with DEPC-treated water. 1% agarose gel was prepared by dissolving 2.5 g of RNase-free agarose in 180 ml of DEPC-treated water in a microwave. To the cooled solution was then added 45 ml formaldehyde and 25 ml 10X MOPS (3-[N-morpholino]-propanesulphonic acid) running buffer (0.2 M MOPS, 0.5 M sodium acetate pH 7.0, 0.01 M EDTA; filtered and wrapped in aluminum foil) and the solution was mixed and replaced in a 60°C waterbath until poured. The gel was submerged in 1X MOPS running buffer.

2.3.2.2 Preparation, electropheresis and transfer of RNA samples
Ten micrograms of ethanol precipitated RNA was centrifuged at 12,000g for 20 minutes at -20°C. The RNA pellet was resuspended in 9 µl of DEPC-treated water and was added to a premix containing 4 µl 10X MOPS running buffer, 7 µl 37% formaldehyde and 20 µl deionised formamide. The mixture was then incubated for 15 minutes at 55°C followed by 5 minutes on ice. 4 µl of formaldehyde loading buffer (1 mM EDTA, pH 8.0/0.25% bromophenol blue/0.25% xylene cyanol/50% glycerol) was added and the samples were run on a pre-electrophoresed gel at a constant 40 volts with buffer recirculation using a peristaltic pump. After the bromophenol blue band had migrated halfway down the gel, RNA was transferred to a Hybond-N nylon membrane by capillary blotting using 20X SSC. RNA was crosslinked to the membrane using a UV stratalinker.
2.3.2.3 Hybridisation and washing the membrane

Blots were prehybridised for a minimum of two hours at 42°C in hybridisation buffer containing 5X SSPE, 0.1% ficoll, 0.1% BSA fraction V, 0.1% polyvinylpyrrolidone, 0.5% SDS, 50% formamide and 50 µg/ml of denatured salmon sperm DNA. Hybridisation with ³²P-labelled murine p58⁸₅TA probe was carried out at 42°C overnight in the above solution. The blots were washed twice in 2X SSPE, 0.1% SDS at 42°C for 15 minutes followed by one wash in 1X SSPE, 0.1% SDS for 30 minutes at 42°C and a final wash in 0.1X SSPE, 0.1% SDS for 15 minutes at room temperature.

2.3.3 RNase protection assay

The RNase protection assay (RPA) was performed using the RPA II kit (Ambion, Austin, Tx) according to manufacturer's instructions. An excess of both the β¹,4-GalTase and control labelled RNA probes were mixed with sample RNA (2.5-10 µg) and carrier RNA to 10 µg total RNA. Ammonium acetate was added to 0.5 M and all RNA species were co-precipitated by adding 2.5 volumes of ethanol, cooling for 15 minutes at -20°C and spinning at 12,000g, 4°C for 20 minutes. The supernatant was carefully removed, the pellet was air dried and resuspended in 20 µl of hybridisation buffer (80% deionised formamide, 100 mM sodium citrate pH 6.4, 300 mM sodium acetate pH 6.4, 1 mM EDTA) by throughly vortexing and briefly centrifuging tubes. The samples were heated at 90°C for 3 minutes, revortexed, briefly recentrifuged and incubated at 4°C overnight to allow hybridisation of the probes to target mRNA contained within the sample RNA. The mixture was then treated with 200 µl of a 1:100 dilution of RNase mixture (250 units/ml RNase A and 10,000 units/ml RNase T1) in RNase digestion buffer for 30 min at 37°C. Protected fragments were precipitated by adding 300 µl of RNase inactivation/precipitation mixture, cooling at -20°C for at least 15 minutes and spinning at 12,000g, 4°C for 20 minutes. The supernatant was then removed completely, the pellet dissolved in 8 µl of gel loading buffer (95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 0.5 mM EDTA, 0.025%
SDS) by vigorous vortexing and brief microfuging. The samples were heated for 3 minutes at 95°C, re-vortexed, re-microfuged briefly and run on a 0.8-mm thick polyacrylamide-urea gel (5% polyacrylamide/1x TBE, 8 M urea) at 250 volts for 1-3 hours depending on the size of the protected fragments. The level of β1,4-GalTase mRNA and of steady state control mRNA or rRNA was assessed either by scanning densitometry of autoradiographs from preflashed Fuji XR film or by scanning the gels using a Bio-Rad GS-250 Molecular Imager (Bio-Rad, Richmond, Ca). Results are expressed as the ratio of β1,4-GalTase mRNA: control signal.

2.3.3.1 Quantitation of mRNA

Initially the level of murine β1,4-GalTase mRNA and of steady state β-actin mRNA was assessed by scanning densitometry of autoradiographs from preflashed Fuji XR film using a Bio-Rad 600 densitometer (Bio-Rad, Richmond, Ca) and by liquid scintillation counting in Aquasol (New England Nuclear, Du Pont, Stevenage, UK) of the radioactivity in the bands excised from the gel (using a Minaxi Tricarb 4000 counter [Packard, Downers Grove, IL]). Later on autoradiographs from preflashed Fuji XR films were analysed using a GS-250 densitometer (Bio-Rad, Richmond, Ca), or alternatively, the level of β1,4-GalTase mRNA and of steady state control RNA was quantified by scanning the gels using a Bio-Rad GS-250 Phospholmager. Results are expressed as the ratio of β1,4-GalTase mRNA: control signal.

Preflashing of films

To increase sensitivity and overcome the non-linear response of film to low intensities of light, Fuji XR films were pre-exposed to an instantaneous flash of light. The optimum positioning of the flashgun (Amersham) was determined by identifying the distance which produced an increase in the absorbance of the developed film of 0.15 OD540 above that of unexposed film. This was done by preflashing the film from various distances, developing and measuring the ODs by placing film sections in a spectrophotometer. The distance of 75 cm
was determined to be optimal and used regularly for pre-flashing films before exposing them to gels.

2.3.3.2 Preparation of radiolabelled size marker

ΦX174 RF DNA HaeIII fragments were labelled using "5' DNA Terminus Labeling System" (Life Technologies) as follows: First, the native 5' phosphoryl group was removed by incubation with bacterial alkaline phosphatase (BAP). The reaction was set up with 10 pmoles (~1.4 µg) of DNA, 1X BAP reaction buffer (supplied in the kit) and 0.5 µl (60 units) of BAP in a total volume of 100 µl and incubated at 65°C for one hour. The reaction mixture was then cooled down to room temperature and extracted twice with an equal volume of phenol-chloroform extraction buffer and once with an equal volume of chloroform. The DNA was precipitated by adding 2.5 volumes of cold ethanol and held at -70°C for 30 minutes. The DNA was collected by centrifugation at 12,000g for 10 minutes and dissolved in 18 µl of ddH₂O. The dephosphorylated DNA was then labelled directly at 37°C for 30 minutes with 5 units of T4 polynucleotide kinase in a reaction containing 1X forward reaction buffer (supplied in the kit) and 10 µCi [γ³²P]-ATP. The labelled DNA was purified using a NucTrap™ push column and stored at -20°C. 500 cpm was loaded on the gel as a size marker in RPAs.

2.3.4 Probes for use in RNase protection assay

2.3.4.1 Murine β 1,4-GalTase

A HindIII-HincII fragment of murine β1,4-GalTase cDNA (Section 2.2.3.1) was subcloned in pBluescript SK⁺ and linearised with Stul to be used as the template for RPA. This was performed as follows: a HindIII-BamHI fragment of murine β1,4-GalTase was removed from pBR322 and cloned in the same sites in pBluescript SK⁺ (see section 2.1.1) E. coli DH5α cells were transformed, colonies were selected on X-gal plates, and analysed by
minipreps. Plasmid DNA obtained from a maxiprep was analysed by digestion with the following restriction endonuclease enzymes: HindIII, BamHI, PstI, SacII and HincII. The band sizes obtained were matched against the restriction map of murine β1,4-GalTase generated using GCG software (the Wisconsin package). HindIII-HincII fragment was then cut out and purified on a low melting temperature agarose gel. The fragment was cloned in the same sites (HindIII-HincII) in pBluescript KS+ and colonies were screened by hybridisation followed by restriction analysis of minipreps. Large amounts of plasmid DNA containing HindIII-HincII fragment of murine β1,4-GalTase was obtained by performing a maxiprep. To make an anti-sense RNA probe, the plasmid DNA was linearised with the enzyme StuI and treated for use in an in vitro transcription reaction. Using T7 RNA polymerase, a probe of 602-nt length would be generated, from which a 559-nt fragment would be protected upon hybridisation to murine β1,4-GalTase mRNA.

2.3.4.2 Human β1,4-GalTase

A SacI-PstI fragment of pgt-2 (Mengle-Gaw et al 1991) was cloned in pBluescript KS+ and linearised with PvuII in order to be used as the template in an in vitro RNA transcription. Pgt-2, provided by Dr L. Mengle-Gaw, was available as a SacI-EcoRI fragment in pUC18. Therefore, E. coli cells were transformed with the plasmid, minipreps were performed and analysed using SacI and EcoRI enzymes. Large amounts of plasmid DNA was obtained by doing a maxiprep with one of the positive cultures. The plasmid DNA was digested with PstI first and then SacI and the resultant 150-bp fragment, purified using a low melting temperature agarose gel, was ligated in PstI-SacI sites of pBluescript KS. E. coli JS5 cells were transformed with the cloned vector and plated out on ampicillin-LB/X-gal plates. Colonies were screened by hybridisation to the human β1,4-GalTase cDNA probe and a few positive colonies were analysed by restriction enzyme digestion of plasmid minipreps. A maxiprep was finally carried out to obtain highly-purified DNA which was linearised with the restriction enzyme PvuII. This would generate a 347-nt
probe upon in vitro transcription using T3 RNA polymerase which would
protect a 150-nt fragment of human β1,4-GalTase mRNA.

2.3.4.3 Control probe: Murine β-actin
Murine β-actin control template (Ambion) was a linearised plasmid containing
a 250 bp mouse β-actin gene fragment in the antisense orientation. The size
of the full length transcript is 350 nt when transcribed with T7 polymerases.

2.3.4.4 Control probes: Murine/Human GAPDH
The GAPDH antisense control template (Ambion) contains a 316-bp
fragment of the mouse or human glyceraldehyde 3-phosphate
dehydrogenase (GAPDH) genes derived from exons 5-8. When transcribed
with T7 RNA polymerase, 404-nt antisense transcripts are produced by this
template.

2.3.4.5 Control probe: Human β-tubulin
The human β-tubulin cDNA clone Db-1, also used as an internal control to
normalise for the amount of RNA in each sample lane, was transcribed with
T3 RNA polymerase from a 264 bp PstI fragment (nucleotides 128-392 of Db-
1 cDNA clone, Mullis et al 1991) subcloned into pBluescript SK⁺ and
linearised with NcoI. The probe was 232-nt long and the fragment protected
by β-tubulin mRNA 172-nt long.

2.3.4.6 Control probe: Human 18S rRNA
pT7 RNA 18S antisense control template (Ambion), also used as an internal
control for the RPA, contains an 80-bp antisense fragment of a highly
conserved region of the human 18S ribosomal RNA (18S rRNA) gene. Under
the transcriptional control of the bacteriophage T7 promoter, the template
produces a 109-nt runoff transcript, 80 nucleotides of which are complementary to human 18S rRNA. 18S rRNA probes were made to a purposefully lower specific activity of approximately $10^3$ cpm/µg using an Ambion T7-MEGAscript™ in vitro transcription kit. 18S rRNA constitutes about 20% of total RNA and for use in RPAs, the probe should be present in a several fold molar excess over the target 18S rRNA. Therefore, high yields of transcription products were needed to use this probe as an internal control in an RPA. This kit provided optimum reaction conditions for RNA synthesis in the presence of high nucleotide and polymerase concentrations. Transcription reactions were set up with 75 mM of all four nucleotides, and only trace amounts of α-32P-rCTP (0.1 µl of 800 Ci/mmol, 10 mCi/ml) in a 20-µl reaction volume and incubated at 37°C for 2 hours. After treating the reaction with 2 units of RNase-free DNase I for 15 minutes at 37°C, the reaction was terminated by adding 115 µl of RNase-free ddH2O and 15 µl of 5 M ammonium acetate, 100 mM EDTA solution. The RNA was then precipitated with one volume of isopropanol, chilled 15 minutes at -20°C and microfuged for 15 minutes at 12,000g. The supernatant was carefully removed and the pellet was resuspended in 0.5 M ammonium acetate, 1 mM EDTA, 0.2% SDS.

2.3.5 Synthesis of radiolabelled RNA probes

2.3.5.1 Preparation of the DNA template

Plasmid DNA to be used as a template in an in vitro RNA transcription reaction was linearised with the desired restriction enzyme and treated with 50 µg/ml proteinase K for 30 minutes at 37°C. The DNA was then phenol extracted, precipitated with two volumes of ethanol, washed in 70% ethanol and dissolved in 10 mM Tris, pH 8.0.
2.3.5.2 *In vitro* RNA transcription reaction

The *in vitro* transcription reaction was carried out using 1 \( \mu g \) of the DNA template and the "RNA Transcription Kit" supplied by Stratagene. The reaction assay included the final concentrations of 40 mM Tris pH 8.0, 8mM MgCl\(_2\), 2 mM spermidine, 50 mM NaCl, 30 mM DTT, 1 unit RNase inhibitor, three unlabelled nucleotides, each at a concentration of 500 \( \mu M \), and a fourth added to a concentration of approximately 3 \( \mu M \) as radioactive ribonucleotide (5 \( \mu l \) of 800 Ci/mmol, 10 mCi/ml \( \alpha^{32P} \)-rCTP). Finally, 10 Units T3 or T7 RNA polymerase was added and the tube incubated for 30 minutes at 37°C. The DNA template was destroyed after the RNA transcription reaction by the addition of 1 unit RNase-free DNase I, for 15 minutes at 37°C.

2.3.5.3 Gel-purification of the probe

To purify the full length RNA transcript, an equal volume of gel loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, 2 mM EDTA) was added to the reaction, heated for 3 minutes at 95°C, and then loaded on a 0.8-mm thick, 8M urea, 5% polyacrylamide gel and run for about one hour at 250 volts. After electrophoresis the gel, on one glass plate, was covered with plastic wrap and exposed to X-ray film for 30 seconds to a few minutes depending on the specific activity of the probe. The film was developed and used to precisely localise the area of the gel that contains the full-length labelled transcript. This small section of the gel was then excised with a scalpel blade, transferred to a microfuge tube, submerged in 350 \( \mu l \) of elution buffer (0.5 M ammonium acetate, 1 mM EDTA, 0.2% SDS) and incubated at 37°C for 2 hours to overnight. The probe, if not used immediately, was stored at -70°C in elution buffer and used within the next two days.

2.3.5.4 Determination of probe specific activity

Before loading the probe on the gel, a 2-\( \mu l \) aliquot was removed and diluted into 498 \( \mu l \) of TE containing 100 \( \mu g \) of yeast tRNA. 5 \( \mu l \) of this dilution was
spotted on a DE81 filter (Whatman International Ltd, Maidstone, UK), air dried and counted directly in a Minaxi Tricarb 4000 counter (Packard, Downers Grove, IL). The filter was washed twice, 15 minutes each time with shaking, in 0.5 M sodium phosphate, pH7.5 and twice in distilled water. The filter was finally washed in ethanol, let to air dry and counted again. The counting efficiency of the $^{32}$P isotope was assumed to be 100%. The specific activity of the RNA probe was calculated as follows:

$$\frac{\text{cpm in washed filter}}{\text{cpm in unwashed filter}} = \text{proportion incorporated}$$

$$\text{proportion incorporated} \times \text{total weight} = \text{total amount of product}$$

$$\frac{\text{cpm incorporated}}{\text{total amount of product}} = \text{specific activity}$$

2.4 IgG concentration and galactosylation

2.4.1 Serum source, collection and storage

2.4.1.1 Source of murine serum

Peripheral blood was taken either by retro-orbital venesection from mice in the middle of studies or by cardiac puncture at the end. The blood was allowed to clot for two hours at 4°C and then microfuged at 2000g at room temperature for 10 minutes. The serum removed and stored in aliquots at -20°C until required.

2.4.1.2 Source of human serum

Peripheral blood was drawn by routine venepuncture and allowed to clot for 30 minutes at 37°C. The samples were then centrifuged at 500g at room temperature for 20 minutes and the serum removed and stored in aliquots at -20°C until required.
2.4.2 Serum IgG concentration

These assays were carried out in collaboration with K. B. Bodman. Serum IgG levels were measured using an ELISA essentially as described by Engvall (1989). Ninety-six well, flat bottomed, maxisorp microtitre plates (Life Technologies) were coated with 100 μl/well of 5 μg/ml affinity purified F(ab')₂ fragment of goat anti-mouse (or human) IgG (Jackson Immunoresearch Laboratories, ME, USA) in PBS and left overnight at 4°C. After washing four times with PBS-0.05% Tween 20 (PBS-T), the plates were blocked with 200 μl/well PBS containing 1% BSA, for one hour at 37°C. Calibrated mouse (The Binding Site, Birmingham Research Park, Birmingham, UK) or human (Behring Diagnostics, London, UK) serum was diluted to a range of 0.001-10 μg/ml of IgG in PBS-T containing 1% goat serum. Supernatant samples were diluted 1:3-5 and sera diluted 1:5000-10,000. The samples and the standards were added in duplicate (100 μl/well) to the washed plates and incubated for one hour at 37°C. After washing with PBS-T, alkaline phosphatase conjugated F(ab')₂ sheep anti-mouse IgG (Sigma) diluted 1:30,000 (or alkaline phosphatase conjugated F(ab')₂ goat anti-human IgG [Sigma] diluted 1:1000) in PBS-T was added to the plates (100 μl/well) and incubated for one hour at 37°C. The plates were then washed four times with PBS-T. A colour reaction was produced using p-nitrophenyl phosphate tablets (Sigma) in carbonate/bicarbonate buffer pH9.6 (2 tablets/10 ml BIC buffer) containing 2 mM magnesium chloride and added at 100 μl/well. The reaction was stopped with 50 μl/well 1M sodium hydroxide and the ODs were read at 405 nm on a Dynatech MR 580 ELISA reader (Dynatech Laboratories Ltd, West Sussex, UK). The immunoglobulin concentration of each sample was interpolated from the standard OD curve plotted on four cycle one-way logarithmic paper.

2.4.3 Serum IgG galactosylation

IgG galactosylation was measured in collaboration with K. B. Bodman. Serum IgG G0 in the human and murine individuals were measured as follows using a modified version of previously published assays (Thompson et al 1992,
Ninety six well maxisorp microtiter plates were coated overnight with 50 µl/well of recombinant truncated protein G' (Sigma, P-4689) at 5.0 µg/ml in PBS at 4°C. The wells were aspirated and blocked with 100 µl 0.05% Tween 20, 1% BSA in PBS (PBS-T-BSA) for one hour at 37°C followed by three washes with 0.05% Tween 20 in PBS. Standards with known G0 levels and sera diluted 1:100 in 0.1 M glycine, 0.16 M NaCl, pH 7.0 were added in triplicate (50 µl/well) to 2 identical plates and incubated for 2 hours at 37°C. After washing, 50 µl/well PBS was added and the plates floated on a waterbath at 85°C for 15 min to partially denature the IgG molecules and thus expose the oligosaccharides. The biotinylated lectin Bandeiraea simplicifolia II (BSII, Vector Laboratories Inc., Cambridgeshire, UK) was diluted 1:500 (4 µg/ml) in PBS-T-BSA containing 0.1 mM calcium chloride and biotinylated goat F(ab')2 anti-human IgG (Sigma) 1:10,000 (0.04 µg/ml) in PBS-T-BSA and each added at 50 µl/well to the cooled plates and incubated at 4°C overnight. After three washes, 50 µl/well of Straptavidin-horseradish-peroxidase (DAKO Ltd., Buckinghamshire, UK) was added and incubated at 37°C for 1 hour. A colour reaction was produced using 50 µl/well 0.1 M citrate phosphate buffer pH 4.1 containing 0.5 mg/ml 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (Sigma) and 1:2000 hydrogen peroxide. After 15 minutes, the reaction was stopped with 50 µl/well sodium fluoride (2 mg/ml) and the plates were read on an automated ELISA reader (Dynatech Laboratories Ltd, Billinghamurst, West Sussex, UK) at 410 nm. The results were expressed as the ratio of BSII:anti-IgG binding and quantified against the standard curve of known G0 samples. The G0 standards used in this study were human (both RA and control subjects) and murine (Balb/c, CBA/Ca, DBA/1 and MRL lpr/lpr) IgG molecules whose G0 values were defined by comparison in both dot blot and ELISA systems with human and murine IgG whose G0, G1 and G2 values had been determined by Dr T Rademacher and colleagues at the Department of Biochemistry, University of Oxford, using the hydrazinolysis method. In some experiments, biotinylated goat F(ab')2 anti-human IgG was replaced by the biotinylated lectin Ricinus communis agglutinin I (RCA I), diluted 1:5000 (1 µg/ml), and the results were expressed.
as the ratio of BSII : RCAI. For measuring galactosylation levels of secreted IgG in cell culture supernatants, protein G' was diluted to 0.05 or 0.20 μg/ml prior to coating and all volumes added to the wells were 100 μl. The concentration of IgG was determined as above in each supernatant (ranged from 0.2-8.0 mg/ml) and serum standard and each diluted to 0.5 or 2.0 μg/ml in glycine buffer. The results were expressed as the ratio of BSII binding to RCAI binding.

2.5 Cell culture

All cell work was carried out under sterile conditions. Cells were cultured in complete medium, unless otherwise stated, and incubated in a 5% CO₂ humidified atmosphere at 37°C. Cells, and supernatants if needed, were harvested by centrifugation at 450g or 350g for human or murine samples respectively.

2.5.1 Culture media

2.5.1.1 Human culture medium

Complete medium consisted of; RPMI 1640 with HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid) supplemented with 10% heat-inactivated FCS (γ-globulin free when IgG galactosylation was to measure; Life Technologies), 2 mM glutamine, 100 units/ml penicillin and streptomycin.

2.5.1.2 Murine culture medium

Complete medium consisted of; RPMI 1640 supplemented with 5% γ-globulin free, heat-inactivated FCS, 2 mM glutamine, 50 units/ml penicillin and streptomycin, 50 nM 2-mercaptoethanol, 1 mM sodium pyruvate and 1% non-essential amino acids (Life Technologies).
2.5.2 Culture with activating agents, cytokines and prolactin

Pokeweed mitogen (PWM, Sigma) or phytohaemagglutinin (PHA, Sigma) were dissolved at 1 mg/ml in distilled water, filtered (0.22 μm) and added separately to the the human PBL cultures at a final dilution of 1 μg/ml. Phorbol myristate acetate (PMA, also called 12-O-tetradecanoylphorbol-13-acetate, TPA, Sigma) was dissolved at 1 mg/ml in absolute ethanol and filtered prior to the addition to the cell cultures at a final dilution of 0.1 μg/ml.

Calcium ionophore (Ionomycin, from *Streptomyces conglobatus*, Sigma) was dissolved at 1mM in dimethylsulphoxide (DMSO) and added to the cell cultures at a final concentration of 1μM. Cyclic AMP (cAMP) inducer, 7β-Acetoxy-1α,6β, 9α-trihydroxy-8,13-epoxy-1abd-14-en-11-one (forskolin, from *Coleus forskohlii*, Sigma) was dissolved at 10 mM in DMSO and added to the human PBL cultures at a final concentration of 20 μM.

Luteotropic hormone (prolactin) (from sheep pituitary glands, 32 I.U./mg, Sigma) was dissolved at 2.5 mg/ml in 0.0001 N NaOH, insulin (from bovine pancreas, Sigma) dissolved at 2.5 mg/ml in 0.01 N Hcl, and Cortisol, 17-hydroxy-corticosterone (hydrocortisone, Sigma) at 8 mg/ml in absolute ethanol. Each reagent was filtered and added to PBL cultures at a final concentration of 5 μg/ml.

Recombinant human IL-2 (100 μg/ml, 4 X 10⁶ units /mg, Genzyme Co., Kent, UK) was added to the enriched peripheral blood B cells at a final concentration of 400 units/ml, anti-human IgM (μ-chain specific)-agarose (4 mg antibody per ml of resin, Sigma) at a final concentration of 1 μg/ml and prolactin (as above) at a final concentration of 100 ng/ml.

Recombinant human interleukin 6 (IL-6, Genzyme, Kent, UK) was added to 250 units/ml and recombinant human tumour necrosis factor-α (TNF-α, Genzyme) to final concentration of 20 ng/ml.
2.5.3 Cell lines

Three IgG-secreting Epstein-Barr virus-transformed B-lymphoblastoid cell lines (2B6, JAC10, and BRAD-3, kindly provided by Dr Belinda Kumpel, International Blood Group Reference Laboratory, Southmead Road, Bristol, UK) were cultured in complete medium for 48 hours at four different concentrations $0.2 \times 10^6$, $0.5 \times 10^6$, $1.0 \times 10^6$, and $2.0 \times 10^6$ cells/ml. Cell viability was determined at the end of incubation period and cells were harvested for RNA analysis.

2.6 Study subjects and mice

2.6.1 Study subjects

Blood samples were obtained from patients with RA, each of whom fulfilled the American Rheumatism Association (ARA) revised criteria for the disease (Arnett et al. 1987). The control population was composed of healthy volunteers. Cells used for in vitro culture were obtained from buffy coats (South Thames Blood Transfusion Service, London).

2.6.2 Mice

2.6.2.1 Mice used in RFLP/mRNA studies

NOD strain mice were obtained from the Clinical Research Centre (Harrow, UK). All other strains of mice (A/J, AKR, C57BL/6, CBA/Ca, DBA/2, MRL +/+; MRL Ipr/Ipr, NZB, NZW and SJL) were purchased at 7 weeks of age from Harlan Olac Ltd (Bicester, UK) and then maintained in the UCL animal facility. 3–5-month-old female mice were used in the initial studies where splenic lymphocytes were isolated as the source of RNA. All the mice used for mRNA analysis of B cells (slgG+) were 12-week-old females. Cell suspensions were prepared from spleen and lymph nodes, from pools of seven mice.
2.6.2.2 Mice used in the study of G0 distribution

The mice used to study the distribution of G0 secreting cells comprised pools of MRL lpr/lpr mice (mean age 4 months, range 3-5 months), and CBA/Ca mice (mean age 5 months, range 3-7 months).

2.6.2.3 Mice used to study the effects of pregnancy on IgG galactosylation

Female Balb/c strain mice (n=22) were purchased at 10 weeks of age from Charles River Ltd, Margate, Kent, UK. Normal 9 week old Balb/c males (n=5) were purchased from the same supplier and used for syngeneic mating in this study. All the animals were maintained on a standard diet and housed one per cage.

2.6.2.4 Transgenic mice

a) IL-6 transgenic mice

C57BL/6 transgenic mice carrying the human IL-6 gene were produced by introducing the human genomic IL-6 gene fused with the human immunoglobulin heavy chain enhancer (Eμ) (Suematsu et al 1989). The mice were bred by Professor Tetsuya Taga and colleagues in the Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan. Mice were sacrificed at 3 months of age, splenic lymphocytes were isolated for the analysis of β1,4-GalTase mRNA and enzyme activity, and sera were obtained for measuring IgG galactosylation levels.

b) TNF-α transgenic mice

CBA x C57BL/6 mice transgenic for the CD2/human TNF-α gene construct (line Tg211, Robert et al 1993) and their littermate controls were bred in our local animal house by Dr J.Tavern and N. Sheikh from mice kindly provided by Dr D. Kioussis and P.Corbella of the National Institute for Medical
Research (London, UK). Mice were bled by cardiac puncture and sacrificed at 19±1 weeks old. Spleen organs were removed, snap-frozen in liquid nitrogen and kept at -70°C until further analysis.

c) CD4 knockout mice

MRL Ipr/lpr mice, homozygous or heterozygous for disrupted CD4 gene, were generated by Dr M. S. Chesnutt and colleagues in San Francisco Veterans Administration Medical Center, San Francisco, Ca. Briefly, CD4-deficient C57BL/6 x 129/SV hybrid mice were produced by homologous recombination in embryonic stem cells with a mutation that interrupts the protein coding region of the CD4 gene (Killeen et al 1993). Homozygous CD4-deficient MRL lpr/lpr mice were then derived through a series of backcrosses between MRL lpr/lpr and CD4-deficient C57BL/6 x 129/SV hybrid mice. MRL lpr/lpr mice, heterozygous for the disrupted CD4 gene, were then produced by backcrossing homozygous CD4-deficient MRL lpr/lpr mice to wild-type MRL lpr/lpr. These mice (which express CD4 normally) were then crossed with homozygous CD4-deficient MRL lpr/lpr mice, so that the CD4-deficient MRL lpr/lpr offspring could be compared with their heterozygous CD4-expressing MRL lpr/lpr littermates. Sera from female mice of two age groups, 4-months and 8-months old, were used in our studies.

2.7 Statistical analysis

Data were expressed as arithmetic means ± standard error of the mean. Comparisons of the means were made using the Student’s t-test for unpaired data. Correlations between variables were analysed using linear regression (least squares method) with Pearson’s coefficient of correlation (r) given. The significance of the analyses is given as the p value.
CHAPTER 3

STRUCTURAL ANALYSIS OF β1,4-GALTASE GENES
3.1 INTRODUCTION

Restriction fragment length polymorphisms (RFLPs) have been used as a tool for genetic analysis since 1974 (Botstein et al. 1980). This technique is based on the properties of restriction endonuclease enzymes which recognise specific short sequences in DNA (usually between four and eight nucleotides in length) and cut the DNA at all such sites, yielding fragments of defined length. Restriction fragments may be separated by electrophoresis in agarose gels according to their molecular size. Fragments encoding specific sequences can then be detected by hybridisation using Southern blotting, in which the DNA from an agarose gel is transferred onto a membrane and hybridised with radioactive probe sequences. This powerful technology makes possible the identification of variants from within a specific region of the genome, using digest of total genomic DNA. Differences amongst individuals in the length of a particular restriction fragment could result from various kinds of genotypic differences: one or more nucleotide bases could differ, resulting in the loss of a cleavage site or the formation of a new one; alternatively, insertion or deletion of blocks of DNA within a fragment could alter its size. These genotypic changes can all be recognised by the altered mobility of restriction fragments on agarose gel electrophoresis.

Since polymorphisms can affect any type of DNA sequence, an alteration producing an RFLP can occur within a coding sequence of a gene, a noncoding sequence (intron), regulatory regions such as promoters and enhancers, sequences between genes, and within repetitive DNA. Estimates are that any two copies of human genome differ at approximately 1 in 200 of all nucleotide positions. This figure is about ten times higher than the proportion of heterozygous nucleotides estimated for protein-coding regions of the genome (about 1 in 2500 base pairs). The difference is not surprising, for it seems likely that protein-coding regions are under more rigid selective pressure, and thus the incidence of mutations in those regions throughout evolution should be lower (Cooper et al. 1985).
These differences in DNA nucleotide sequences (RFLPs) are inherited in a Mendelian co-dominant manner and the majority are apparently without any phenotypic effects as they usually occur in intergenic DNA. Each can, however, be of value as a genetic "marker" for that point of the DNA molecule. Thus RFLPs have been used in the analysis of gene structure, genetic mapping by linkage analysis, presymptomatic and prenatal diagnosis of genetic diseases, detection of heterozygous carriers of genetic disease, paternity testing and forensic applications, matching of donor-receptor pairs for tissue and organ transplantation, and finally evaluation of high- and low-risk persons with a predisposition to common adult disorders such as coronary heart disease, cancer and diabetes (Mueller and Young 1995).

As discussed earlier, decreased levels of IgG galactosylation in patients with RA and in the animal model of this disease, MRL lpr/lpr, have been associated with aberrant regulation of the enzyme β1,4-GalTase. In this chapter, we asked if this could be due to structural alterations in the β1,4-GalTase and/or putative regulatory p58GTA gene loci. Thus, the structural integrity of β1,4-GalTase and p58GTA genes were assessed using restriction endonuclease digestion of DNA from patients with RA and from several autoimmune and non-autoimmune strains of mice including MRL lpr/lpr.
3.2 RESULTS

3.2.1 Human studies

RFLP of human \( \beta 1,4\)-GalTase

In an attempt to find possible disease-specific alterations in the structure of the \( \beta 1,4\)-GalTase gene, DNA from RA patients was analysed for the occurrence of RFLPs and compared to DNA isolated from normal subjects (Table 3.1).

Table 3.1 Restriction fragment length polymorphism of the \( \beta 1,4\)-GalTase gene.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Band Sizes (Kb)</th>
<th>Samples Analysed</th>
<th>Polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>SacI</td>
<td>14, 11, 8.0, 7.0, 5.5</td>
<td>20 RA, 19 Normal</td>
<td>Homozygous and Heterozygous 11-kb and 8-kb bands</td>
</tr>
<tr>
<td>EcoRI</td>
<td>6.5, 5.5, 4.5, 3.3</td>
<td>6 RA, 22 Normal</td>
<td>5.5 Kb band absent in some individuals</td>
</tr>
<tr>
<td>HindIII</td>
<td>(9.4), 6.7, 6.5, 2.0</td>
<td>10 RA, 5 Normal</td>
<td>None detected</td>
</tr>
<tr>
<td>BglII</td>
<td>&gt;23, 10</td>
<td>6 RA, 20 Normal</td>
<td>None detected</td>
</tr>
</tbody>
</table>

The enzyme SacI detected a polymorphism in the \( \beta 1,4\)-GalTase gene locus when a mixture of five probes were used (Figures 3.1 and 3.2). The 8-kb and 11-kb bands revealed subjects who were either homozygous or heterozygous for this polymorphism, although no statistically significant difference in the occurrence of these three genotypes was observed between 20 RA patients and 19 normal controls, possibly due to the relatively small number of samples analysed (Table 3.2).
**Figure 3.1** Restriction map of the human β1,4-GalTase locus. Filled boxes delineate coding sequences and the hatched region shows 3' untranslated sequence. Restriction enzyme sites are indicated as follows: R=EcoRI, S=SacI, X=Xbal. The probes corresponding to this genomic locus which are used in this study are shown underneath the map (open boxes). The probe sizes are: pgt1 (350 bp), pgt2 (1.7 kb), pgt3 (800 bp), pgt4 (750 bp), and pgt6i (1.9 kb).

**Figure 3.2** Southern blot analysis of SacI-digested DNA from normal individuals (lanes 1-9) and from patients with RA (lanes 10-17) hybridised with a mixture of five β1,4-GalTase genomic probes. Molecular size markers are shown at the left.
Table 3.2  Frequencies of the 11-kb and 8-kb SacI RFLP in the β1,4-GalTase gene from 20 RA patients and 19 controls. No statistically significant difference in allele frequency between patients and controls was identified by chi-squared test.

<table>
<thead>
<tr>
<th>RFLP</th>
<th>Genotype</th>
<th>No. of patients</th>
<th>No of controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 kb</td>
<td>homozygous</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>11 kb</td>
<td>homozygous</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>8 kb + 11 kb</td>
<td>heterozygous</td>
<td>10</td>
<td>6</td>
</tr>
</tbody>
</table>

Rehybridisation of the blots with individual probes separately localised this allelic variation to the segment of the gene locus hybridising to the pgt2 and pgt6i probes (Figure 3.3). To further localise the polymorphism to the exon or intron of the genome, DNA from homozygous and heterozygous individuals was digested with SacI and XbaI (the XbaI site lies just outside the exon 2 as seen in Figure 3.1) and hybridised to the pgt2 or pgt6i probe (Figure 3.4). All three individuals showed one band of about 2.5-kb size on filters hybridized with pgt2, indicating that the polymorphism lies in the intron-2 of the gene locus. This was supported by the observation of two bands of approximately 7.5- and 4.5-kb on filters hybridized with pgt6i for the heterozygous individual, and one band of either 7.5- or 4.5-kb for the homozygous individuals.

The use of the enzyme EcoRI revealed bands of ~6.5-, 5.5-, 4.5- and 3.3-kb sizes, with the 5.5-kb band missing in one out of six RA patients and eight out of twenty two controls (Figure 3.5). A very weak band of ~ 5.0-kb was present in some of the lanes missing the 5.5-kb band which had more DNA loaded.

Two restriction enzymes, HindIII and BglII showed similar band patterns in all the RA and normal subjects tested. A representative example using BglII is shown in Figure 3.6.
Figure 3.3 Southern blot analysis of Sacl-digested DNA hybridised with pgt2 probe only. Lanes 1, 2 and 4, DNA from individuals heterozygous for 8-kb and 11-kb bands. Lane 3, DNA from an individual homozygous for 11-kb band. Lane 5, DNA from an individual homozygous for 8-kb band. Molecular size markers are shown at the left.
Figure 3.4  Southern blot analysis of double digested DNA (SacI and XbaI) hybridised with the pgt2 probe only. Lane 1, DNA from an individual homozygous for the 8-kb band. Lane 2, DNA from an individual homozygous for the 11-kb band. Lane 3, DNA from an individual heterozygous for 8-kb and 11-kb bands. Molecular size markers are shown at the left.
Figure 3.5  Southern blot analysis of EcoRI digested DNA from normal individuals hybridised with a mixture of the five β1,4-GalTase genomic probes. Molecular size markers are shown at the left.

Figure 3.6  Southern blot analysis of BglII-digested DNA from normal individuals (lanes 1-8 and 12) and from RA patients (lanes 9-11) hybridized with the five β1,4-GalTase genomic probes. Molecular size markers are shown at the left.
3.2.2 Murine studies

Analysis of IgG galactosylation in autoimmune-prone and nonautoimmune-prone strains of mice showed that the percentage of serum IgG oligosaccharides with exposed GlcNAc residues, and therefore lacking galactose residues, varied between different strains of inbred mice in two-month old females (Figure 3.7). MRL \textit{lpr/lpr} mice of this age had the highest levels of exposed GlcNAc residues (55.2%) indicating a lower level of IgG galactosylation than in any of the other strains tested (Bodman \textit{et al} 1994).

![Figure 3.7](image)

\textbf{Figure 3.7} Mean serum G0 levels ± standard error of the mean in eleven strains of mice (\(n = 3\) per group) at two months of age. The %G0 levels were measured by K. Bodman.

3.2.2.1 RFLP of murine \(\beta1,4\)-GalTase

Using a panel of 10 restriction endonucleases (\textit{BamHI}, \textit{BglII}, \textit{EcoRI}, \textit{HincII}, \textit{HindIII}, \textit{MspI}, \textit{PvuII}, \textit{RsaI}, \textit{SacI} and \textit{TaqI}) no polymorphisms were detected for the gene encoding \(\beta1,4\)-GalTase in any of 11 strains of autoimmune-prone and nonautoimmune-prone mice (A/J, AKR, C57BL/6, CBA/Ca, DBA/2, MRL +/+, MRL \textit{lpr/lpr}, NOD, NZB, NZW and SJL) (A representative example...
using the enzyme \textit{HincII} is shown in Figure 3.8 and the full results are summarised in Table 3.3). Thus, this gene locus would appear to have a similar organisation in all the murine strains examined, including the MRL \textit{lpr/lpr} strain which shows reduced IgG galactose levels.

3.2.2.2 RFLP of murine p58\textsuperscript{GTA} protein kinase gene

Eleven autoimmune-prone and nonautoimmune-prone strains of mice were analysed for RFLPs using ten different restriction endonucleases as described above. For each of the endonucleases, one of two alternative band patterns was observed when Southern blots were hybridised with a cDNA probe for the p58\textsuperscript{GTA} protein kinase gene. Thus, for each endonuclease enzyme, an identical pattern was seen for NOD and SJL strain mice whilst a different pattern was seen in the other strains tested. This strongly suggests that NOD and SJL share an allelic variant “b” of the p58\textsuperscript{GTA} gene locus which substantially differs from allele “a” found in the other nine strains of mice examined, including MRL \textit{lpr/lpr}. A representative example using the enzyme \textit{HincII} is shown in Figure 3.9 and the full results are summarised in Table 3.3.

Both allelic variants a and b encode a full length p58\textsuperscript{GTA} mRNA as assessed by Northern blot analysis (Figure 3.10), and the SJL and NOD strains which possess the less common allele “b” are able to galactosylate their IgG oligosaccharides (Figure 3.7).

Structure of the p58\textsuperscript{GTA} gene was also investigated in two strains genetically related to the NOD mouse; NON (nonobese nondiabetic) and CTS (cataract Shinogi) strain mice. All the three strains have been derived from a cataract mouse found in outbred ICR mice (Kikutani and Makino 1992). As shown in Figure 3.11, the NON and CTS strains, however, possess the common allelic variant “a” of the p58\textsuperscript{GTA} gene.
Southern blot analysis of HincII-digested liver DNA from different mouse strains hybridised with the β1,4-GalTase cDNA probe. Molecular size markers (bacteriophage λ DNA digested with HindIII) are shown at the left.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Kb</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/J</td>
<td>6.7</td>
</tr>
<tr>
<td>SJL</td>
<td>4.4</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>2.3</td>
</tr>
<tr>
<td>AKR</td>
<td>2.0</td>
</tr>
<tr>
<td>CBA/Ca</td>
<td></td>
</tr>
<tr>
<td>MRL-1pr/1pr</td>
<td></td>
</tr>
<tr>
<td>MRL-+/+</td>
<td></td>
</tr>
<tr>
<td>MRL-1pr</td>
<td></td>
</tr>
<tr>
<td>MOD</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.8
Figure 3.9  Southern blot analysis of HincII-digested liver DNA from different mouse strains hybridised with the p58\(^{GA}\) cDNA probe. Molecular size markers (bacteriophage \(\lambda\) DNA digested with HindIII) are shown at the left.
Table 3.3  Summary of Southern blot analysis of the β1,4-GalTase and p58\textsuperscript{GTA} protein kinase genes in 11 strains of mice.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>β1,4-GalTase (all strains)</th>
<th>p58\textsuperscript{GTA} (NOD &amp; SJL mice)</th>
<th>p58\textsuperscript{GTA} (all the other strains)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI</td>
<td>16.0, 3.6**</td>
<td>(6.0), 4.4, 2.7, 2.4, 1.6</td>
<td>(11), 5.5, (4.8), 4.3, 2.4, 1.6, 0.96</td>
</tr>
<tr>
<td>BglII</td>
<td>7.8, 5.4, 1.6</td>
<td>7.0, 6.7, 3.0, 0.7</td>
<td>7.0, 2.9, 0.7</td>
</tr>
<tr>
<td>EcoRI</td>
<td>16.5, 4.2, 3.7</td>
<td>7.4, 4.9(d), 2.6, 2.4</td>
<td>7.4, 4.9, 2.4, 2.0</td>
</tr>
<tr>
<td>HincII</td>
<td>4.6, 2.2, 1.9, 1.5</td>
<td>6.7, 4.6, 3.0, 2.3, 1.6</td>
<td>7.2, 3.5, 3.0, 2.3, 1.6</td>
</tr>
<tr>
<td>HindII</td>
<td>&gt;23, 2.2</td>
<td>10.0, 4.7, 4.1, 3.3, 1.2</td>
<td>9.4, 4.5, 4.3, 3.3, 1.2</td>
</tr>
<tr>
<td>MspI</td>
<td>3.9, 3.4, 3.0, 2.8, 2.6</td>
<td>3.7, 3.4, 2.8, 2.5, 2.0</td>
<td>4.2, 3.8, 2.0</td>
</tr>
<tr>
<td>(1.6), (1.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PvulII</td>
<td>2.3, 2.1, 1.8</td>
<td>6.3, 1.9, (1.0), 0.9, 0.8, (0.7)</td>
<td>3.4, (3.3), 1.7, (1.0), 0.9, 0.8, (0.7)</td>
</tr>
<tr>
<td>RsaI</td>
<td>1.9, 1.1(d), (0.8)</td>
<td>2.3, 0.78</td>
<td>2.0, 0.88, 0.78</td>
</tr>
<tr>
<td>SacI</td>
<td>5.6, 3.2, 1.4</td>
<td>10.4, 4.4, 2.5, 1.2, &lt;0.6</td>
<td>10.4, 4.4, 3.7, 1.2, &lt;0.6</td>
</tr>
<tr>
<td>TaqI</td>
<td>4.8, 2.3</td>
<td>7.0, (3.5, 3.2, 2.9, 2.5), 2.2, 1.8</td>
<td>4.0, (3.5, 3.2, 2.9, 2.5), 2.2, 1.8</td>
</tr>
</tbody>
</table>

* = Mouse strains analysed were A/J, AKR, C57BL/6, DBA/2, MRL +/+ , MRL lpr/lpr, NOD, NZB, NZW and SJL.

** = All band sizes are in kilobases.

(d) = Doublet.

( ) = Bands in parenthesis were only weakly visible on Southern blots.
Figure 3.10  Northern blot analysis of total RNA isolated from liver of four different strains of mice and hybridised with the pS8\(^{GTA}\) cDNA probe.
Figure 3.11 Structure of the p58GTA gene in the NOD mouse and related strains NON and CTS.
3.3 DISCUSSION

The RFLP studies with genomic probes for human \( \beta 1,4\)-GalTase showed that, like many other gene loci, this gene locus is also polymorphic in man. The use of the enzyme SacI revealed that the \( \beta 1,4\)-GalTase locus exists as different polymorphic alleles, as shown by the homozygosity and heterozygosity of the 11-kb and/or 8-kb bands. Further investigation showed that the variation lies outside the coding region, in intron 2 of the gene. Whether this region of the locus is involved in regulation of gene expression remains to be determined. The SacI polymorphism was found both in normal individuals and in patients with RA, with no significant difference in frequency between these two groups. The enzyme EcoRI revealed an additional polymorphism with some individuals missing a 5.5-kb band and possessing a faint band of 5.0-kb size, although this weak band was only visible in lanes containing higher amounts of DNA. The other two enzymes, HindIII and BglII, did not detect any polymorphism at all.

In another study (Dalziel and Axford 1994), possible polymorphism in the \( \beta 1,4\)-GalTase gene was investigated using 10 different restriction enzymes, in combination with two probes; a 1.28-kb cDNA probe specific for the coding region and a 0.2-kb probe specific for the 5' untranslated region. Only one restriction enzyme, Psfl, was reported to detect a polymorphism in one out of ten controls and one out of ten patients with RA. Therefore, although the \( \beta 1,4\)-GalTase gene is present in different allelic forms in human, none of the reported polymorphisms are specifically associated with RA.

Possible structural alterations in the human p58\(^{GTA}\) protein kinase gene was investigated previously by Delves and colleagues. It was reported that the gross structure of the p58\(^{GTA}\) protein kinase gene locus remains intact in patients with RA. This gene locus is also polymorphic both in normal individuals and in patients with RA, although no polymorphisms unique to RA patients were observed (Delves et al 1990).
In murine studies, the β1,4-GalTase gene was found to have a similar structure in each of 11 different inbred strains using a cDNA probe containing 1.6 kb of the murine β1,4-GalTase locus. MRL lpr/lpr mice, which possess high levels of agalactosyl IgG in serum, showed no gross structural alterations of the β1,4-GalTase gene. The p58GTA gene locus, however, showed an allelic variation in two autoimmune-prone strains of mice. Although apparently encoding a full length mRNA, the overall structure of the p58GTA protein kinase gene locus appeared to be very different in NOD and SJL mice compared to the other strains tested. It is not known if these differences in any way affect putative regulatory regions of the gene. NOD strain mice spontaneously develop both a Sjögrens-like syndrome and a polyendocrine autoimmunity which includes pancreatic and thyroid autoimmune disease (Kikutani et al 1992, Bernard et al 1992). SJL strain mice, whilst not developing spontaneous autoimmune disease, are particularly susceptible to induced autoimmune diseases, possibly due to defective suppression of autoantibody responses (Elliot and Cooke 1992). It is also of note that SJL mice develop a non-autoimmune insulin-dependent diabetes mellitus after inoculation with encephalomyocarditis EMC-D virus or reovirus type 1 (Bae et al 1989, Onodera et al 1978). Although our data indicates that the different structure of p58GTA protein kinase gene does not abolish IgG galactosylation in NOD and SJL mice, any alteration in the regulation of the p58GTA gene could have other implications for cellular dysfunction in these autoimmune-prone strains. In addition to β1,4-GalTase activation, this protein kinase is able to phosphorylate histone H1 and casein, suggesting a range of substrate specificities, and its over-expression leads to a prolonged late telophase/early G1 phase of the cell cycle (Bunnell et al 1990). The p58GTA gene locus in the non-diabetic but genetically related CTS and NON strains (Kikutani and Makino 1992) shows the same structure to that found in the majority of strains tested. Therefore, even if it turns out to be of no functional significance, the polymorphism of this gene locus in SJL and
NOD mice provides an additional marker for genetic analysis of these autoimmune-prone strains.

Rheumatoid arthritis and most other autoimmune diseases have multifactorial and/or polygenic aetiology. Unravelling the molecular genetics of such complex multifactorial diseases is much more difficult than for single gene disorders. Identification of disease associations in the general population, linkage analysis in affected families, and the study of animal models are usually the approaches taken in trying to understand multifactorial disorders. Studies on the structural analysis of both the β1,4-GalTase (present study and Dalziel et al 1994) and p58GTA (Delves et al 1990) genes in human and also in an animal model of RA, MRL lpr/lpr (present study) have not found any associations between β1,4-GalTase and p58GTA genetic polymorphisms and RA. Sequencing the polymorphic region(s) of the β1,4-GalTase and the p58GTA protein kinase gene loci will reveal the structural basis for the polymorphisms and show if the RFLPs affect the binding sites of the enzyme or the regulatory regions of the gene. More detailed analysis of the human SacI polymorphism in the present study showed that this RFLP did not lie in the coding region of the gene. Whether this part of the second intron of β1,4-GalTase is involved in the regulation of gene expression remains to be shown.

In conclusion, the results of the RFLP analyses presented here, taken together with the fact that normal galactosylation is re-established during disease remission in patients with RA, would argue against a defective allelic variant of either β1,4-GalTase or the regulatory p58GTA protein kinase being associated with the reduced IgG galactosylation seen in rheumatoid arthritis.
CHAPTER 4

EXPRESSION OF THE β1,4-GALTASE GENE
4.1 INTRODUCTION

It has been suggested that defective galactosylation of IgG may be due to aberrant control of lymphocytic β1,4-GalTase activity. A reduction in the activity of β1,4-GalTase enzyme has been detected in patients with RA as discussed earlier (see section 1.3.2.1). Since glycosyltransferases, in general, are thought to be regulated at the transcriptional level, we have measured the mRNA levels for the β1,4-GalTase enzyme in B cells (CD19+) from RA patients and compared them to these in normal individuals. In parallel studies, in mice, mRNA levels in B cells (surface IgG positive, sIgG+) in the spleen and lymph nodes were measured.

The Ribonuclease Protection Assay (RPA) was chosen to measure and compare the level of β1,4-GalTase mRNA in the different test samples. RPA is an extremely sensitive procedure for the detection and quantitation of RNA species in a complex sample mixture of total cellular RNA. Compared to hybridisation protocols that rely on RNA bound to solid support (i.e. Northern blots), low abundance mRNAs are detected more readily and quantified more accurately by using a solution hybridisation procedure such as the RPA (Lee and Costlow 1987). Since the probes used in the RPA are generally significantly shorter than the mRNA species being detected, the target RNA preparation need not be completely intact (breaks in mRNA that occur outside the region that hybridises to the probe will have no effect on the RPA, but will result in loss of signal on Northern blots). Another technique which is being used more recently for quantitative analysis of mRNA expression is the polymerase chain reaction (PCR), a powerful method for amplifying specific DNA sequences in vitro. By reverse transcribing mRNA into cDNA, it is possible to detect and amplify rare mRNA transcripts through PCR amplification, although it is more difficult to quantitate the amount of mRNA present in the starting material. This is because the efficiency of the amplification process is greatly affected by minute differences in any of the variables that control the reaction rate (Wang et al 1989). Although the
quantitation strategy in PCR has been improved considerably since we started these experiments, setting up a reliable and reproducible quantitative PCR still requires a great deal of effort and the determination of the amount of total RNA or the number of cells in the original sample. Therefore, in spite of being difficult to set up and optimise, the RPA we have used offers several advantages over other assays for measuring gene transcription levels.

It is known that human peripheral blood B cells are capable of secreting agalactosyl IgG (Bodman et al. 1992). Thus, this cell population provides one appropriate source of cells for exploring the relationship between β1,4-GalTase mRNA expression and IgG glycosylation. However, the small blood volume of the mouse makes this cell source problematical for studies in this species. Therefore, larger B cell compartments, i.e. spleen and lymph nodes, were used for measuring transcript levels in the murine studies. To establish that these compartments contribute to the high level of agalactosyl IgG detected in the serum of MRL lpr/lpr mice, and thus provide a relevant source of B cells, cell suspensions from peripheral blood, bone marrow, lymph nodes and spleen of these mice were analysed for spontaneous secretion of agalactosyl IgG. CBA/Ca strain mice were used as the control for comparison in these experiments. This study was done in collaboration with Katherine Bodman.

Furthermore, β1,4-GalTase mRNA levels were compared in three cell lines with different β1,4-GalTase enzyme activity levels. Galactosylation of human IgG monoclonal anti-rhesus D produced by different EBV-transformed B-lymphoblastoid cell lines has been analysed as part of a study by Kumpel and colleagues. The monoclonal antibodies, 2B6 (IgG1), JAC10 (IgG1), and BRAD-3 (IgG3), all specific for the Rh D antigen on human erythrocytes, were produced in serum-free Iscove's modified DMEM using cells cultured at 2.3-2.9x10^7 per ml. Biochemical analysis of asparagine-linked oligosaccharides released from these antibodies revealed a remarkably high level of digalactosyl (71-79% G2) chains with extremely low levels of agalactosyl
(2-5% G0) oligosaccharide chains. The levels of β1,4-GalTase enzyme activity measured showed differences between the three cell lines with the highest β1,4-GalTase activity in the 2B6 cell line (131±12 pmol/min/mg) followed by JAC10 (109±11 pmol/min/mg), and the lowest activity in the BRAD-3 (51±7 pmol/min/mg) cell line. This range of values contrasted with equivalent galactose content for these three monoclonal antibodies (Kumpel et al 1994).

In the present study, the mRNA levels for the β1,4-GalTase enzyme were measured in 2B6, JAC10, and BRAD-3 cell lines to see if the levels of β1,4-GalTase gene expression correlate with the levels of enzyme activity in these cell lines. In addition, the effect of changes in cell density on β1,4-GalTase mRNA was also studied.
4.2 RESULTS

4.2.1 Human studies

4.2.1.1 IgG galactosylation levels in patients with RA

Studies on the comparison of β1,4-GalTase mRNA levels in RA and normal individuals were carried out using three different cell populations; PBMCs (total cells isolated using lymphoprep), PBLs (PBMCs subjected to plastic adherence and non-adherent cells selected), and B cells (CD19⁺ selected). The percentage of oligosaccharide chains lacking galactose was significantly higher in the sera from the patients with RA than in the normal subjects in all three experiments performed (Figure 4.1).

![Figure 4.1](image)

Figure 4.1 Percentage of agalactosyl IgG in sera of RA patients and of normal individuals in three separate studies. Mean ± standard error of the mean is indicated. G0 levels were measured by K. Bodman.
4.2.1.2 β1,4-GalTase mRNA levels in patients with RA

β1,4-GalTase mRNA levels in patients with RA and controls were compared in three different cell populations:

i. β1,4-GalTase mRNA levels were measured in PBMCs and found to be reduced significantly in 8 patients with RA compared to 8 normal controls (p<0.005). This was true with measuring β1,4-GalTase mRNA levels in comparison with two different probes (18S rRNA and β-tubulin) used as internal control in the RPA (Figure 4.2a,b and 4.3a,b).

ii. The level of β1,4-GalTase mRNA in peripheral blood lymphocytes from 6 patients with RA and 6 normal individuals was found to be comparable (p>0.9, 4.4a,b).

iii. The level of β1,4-GalTase mRNA was measured in B cells (CD19+ cells) from 14 patients with RA and 13 normal controls and found to be similar (p>0.7, Figure 4.5a,b). No relationship was found between the level of IgG galactosylation and the level of β1,4-GalTase mRNA in CD19+ B-cells (r=0.27, p>0.2).
Figure 4.2a PBMC β1,4-GalTase mRNA levels in normal individuals and in patients with RA using RPA with 18S rRNA as an internal control.

Controls: Lane A, yeast tRNA hybridised with 18S rRNA in the absence of RNase, showing full length 109 nt 18S rRNA probe. Lane B, yeast tRNA hybridised with β1,4-GalTase in the absence of RNase, showing full length 347-nt β1,4-GalTase probe. Lane C, yeast tRNA hybridised with 18S rRNA probe but with addition of RNase. Lane D, yeast tRNA hybridised with β1,4-GalTase probe in the presence of RNase. β1,4-GalTase mRNA is protected by a 150-nt fragment and 18S rRNA by a 82-nt fragment.
Figure 4.2b  PBMC β1,4-GalTase mRNA levels in normal individuals and in patients with RA using RPA with β-tubulin as an internal control.

Controls: Lane A, yeast tRNA hybridised with both β1,4-GalTase and β-tubulin probes in the presence of RNase. Lane B, tRNA hybridised with β-tubulin but without RNase, showing full length 232-nt β-tubulin probe. Lane C, tRNA hybridised with β1,4-GalTase in the absence of RNase, showing full length 347-nt β1,4-GalTase probe. β1,4-GalTase mRNA is protected by a 150-nt fragment and β-tubulin by a 170-nt fragment.
Figure 4.3  Quantitation of the human PBMC β1,4-GalTase mRNA levels by densitometry using 18S rRNA (a) or β-tubulin (b) as an internal control. Results are expressed as the ratio of β1,4-GalTase mRNA signal over 18S rRNA signal (a) or as the ratio of β1,4-GalTase mRNA signal over β-tubulin mRNA signal (b). Mean ± standard error of the mean is indicated.
Figure 4.4a  β1,4-GalTase mRNA levels in PBLs from normal individuals (1-6) and patients with RA (7-12) using RPA with β-tubulin as an internal control (5 and 10 μg of total RNA was used).

Controls: Lane A, yeast tRNA hybridised with both β1,4-GalTase and β-tubulin probes without RNase, showing full length 347-nt β1,4-GalTase probe and 232-nt β-tubulin probe. Lane B, human lymphocyte RNA hybridised with β1,4-GalTase with addition of RNase, showing 150-nt protected fragment. Lane C, yeast tRNA hybridised with β-tubulin probe without RNase, showing full length 232-nt β-tubulin probe. Lane D, yeast tRNA hybridised with β-tubulin probe with addition of RNase. Lane E, yeast tRNA hybridised with β1,4-GalTase probe with addition of RNase.
Figure 4.4b  Quantitation of the human PBL β1,4-GalTase mRNA levels by densitometry using β-tubulin as an internal control. Results are expressed as the ratio of β1,4-GalTase mRNA signal over β-tubulin mRNA signal. Mean ± standard error of the mean is indicated.
Figure 4.5a A phospho-image of β1,4-GalTase mRNA levels in peripheral blood B-lymphocytes (CD19\(^+\) cells) from normal individuals and patients with RA using RPA with 18S rRNA as an internal control.

Controls: Lane 10, yeast tRNA hybridised with both β1,4-GalTase and 18S rRNA probes with the addition of RNase. Lane 11, human B-lymphocyte (CD19\(^+\)) RNA hybridised with 18S rRNA probe only, showing the protected fragments (doublet). Lane 12, human B-lymphocyte (CD19\(^+\)) RNA hybridised with β1,4-GalTase probe only, showing the protected fragment. Lane 13, yeast tRNA hybridised with β1,4-GalTase probe only without RNase to show the full length probe for β1,4-GalTase (which is not seen in the captured image, but it was present on the full image). Lane 14, yeast tRNA hybridised with 18S rRNA probe only without RNase, showing the full length probe for 18S rRNA.
Figure 4.5b  Quantitation of the human peripheral blood B cell (CD19+) β1,4-GalTase mRNA levels by phospho-imaging. Because of limited amounts of RNA, some B cell RNA samples were pooled from two different individuals with similar levels of IgG G0 (these are shown using the symbol triangle). Results are expressed as the ratio of β1,4-GalTase mRNA signal over 18S rRNA signal. Mean ± standard error of the mean is indicated.
4.2.2 Murine Studies

4.2.2.1 β1,4-GalTase gene expression in splenic lymphocytes from MRL lpr/lpr mice

Lymphocytic (i.e. splenic cells subjected to plastic adherence) β1,4-GalTase mRNA levels were measured in four different strains of mice (MRL lpr/lpr, MRL +/+ , SJL and CBA/Ca). The results of an experiment comparing MRL lpr/lpr with CBA/Ca strain mice are shown in figure 4.6. Visually, differences were seen most obviously when lanes with comparable levels of loading, such as MRL lpr/lpr lane 3b and CBA/Ca lane 3a, were compared. In order to compensate for different amounts of RNA between lanes, results were quantified as the ratio of the signal (peak area on the case of densitometric analysis and cpm for scintillation measurements) obtained for β1,4-GalTase mRNA over β-actin mRNA. The values obtained by both methods were lower for all three 5-month-old female MRL lpr/lpr mice compared to those for age- and sex-matched CBA/Ca mice (Table 4.1; p= 0.002, unpaired Student's t-test) indicating a reduced level of β1,4-GalTase mRNA expression relative to β-actin mRNA expression in MRL lpr/lpr compared to CBA/Ca strain mice.

The data obtained from five separate experiments comparing the levels of β1,4-GalTase mRNA between four different strains are shown in Figure 4.7. Since the specific activity of the probes made on different occasions were not identical, the results (signal intensity) obtained were not directly comparable from experiment to experiment. Therefore, the mean value of the mRNA levels obtained in each experiment for the CBA/Ca strains was arbitrarily assigned a value of 100 and the results for the other strains were expressed in relative proportion to that of CBA/Ca mice used in that experiment. As seen in the Figure 4.7, in contrast to MRL lpr/lpr mice, the genetically similar MRL +/+ mice had levels of β1,4-GalTase mRNA that were comparable to those seen in CBA/Ca strain mice. The SJL strain mice had levels of β1,4-GalTase mRNA that were intermediate between CBA/Ca and MRL lpr/lpr.
Figure 4.6 Splenic lymphocyte β1,4-GalTase mRNA levels in 5-month-old female MRL lpr/lpr and CBA/Ca mice using RPA.

Controls: Lane A, murine spleen RNA hybridized with β1,4-GalTase probe alone to show 559-nt protected fragment. Lane B, murine spleen RNA hybridized with β-actin probe alone to show 250-nt protected fragment. Lane C, yeast tRNA hybridized with both probes but without RNase, showing full length 602-nt β1,4-GalTase probe and 350-nt β-actin probe. Lane D, as lane C but with addition of RNase A and RNase T1. "a" and "b" are duplicate lanes of RNA from the same mouse showing the 559-nt (β1,4-GalTase) and 250-nt (β-actin) protected fragments.
Table 4.1  Quantitation by two different methods of the RPA results from the experiment presented in figure 4.6.

<table>
<thead>
<tr>
<th></th>
<th>MRL /pr/pr strain mice</th>
<th>CBA/Ca strain mice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lane</strong></td>
<td>1a 1b 2a 2b 3a 3b</td>
<td>1a 1b 2a 2b 3a 3b</td>
</tr>
<tr>
<td>Densitometry</td>
<td>0.59 0.85 0.77 0.84 0.46 0.64</td>
<td>1.55 1.86 1.64 1.61 2.05 2.07</td>
</tr>
<tr>
<td>Scintillation</td>
<td>0.43 0.56 0.79 0.50 0.39 0.48</td>
<td>0.94 1.48 1.29 1.41 1.27 1.69</td>
</tr>
</tbody>
</table>

Figure 4.7  β1,4-GalTase /β-actin mRNA levels in splenic lymphocytes from four strains of mice. The levels from five separate experiments are normalised against the mean value (arbitrarily assigned a value of 100) obtained in each experiment for the CBA/Ca strain.
4.2.2.2 IgG glycosylation in the different strains of mice

Levels of IgG galactose, derived from a standard curve of IgG of known galactose content, were measured (by K. Bodman) in sera obtained from mice at the same time as the spleens were removed for the mRNA measurements. The percentage of chains lacking galactose was greatest in MRL /pr/7pr strain mice. The reduced level of GalTase mRNA seen in splenic lymphocytes from the MRL lpr/lpr mice, therefore, correlated (r = 0.86, p = 0.026) with the increased level of IgG exposed GlcNAc in the same mice (Figure 4.8). In one of the six MRL lpr/lpr mice there was a higher level of β1,4-GalTase message, and this was associated with higher levels of IgG galactose. Although the SJL strain mice showed lower levels of β1,4-GalTase mRNA than MRL +/+ and CBA/Ca, the reduction in mRNA levels was not as profound as in the MRL lpr/lpr mice, and was not significantly associated with reduced levels of galactose on serum IgG.

**Figure 4.8** Correlation between percentage serum IgG lacking galactose and β1,4-GalTase mRNA levels. (□) MRL lpr/lpr; (●) SJL; (△) MRL +/+; (■) CBA/Ca. Although 10 CBA/Ca mice are included in the study, they appear as a single data point (i.e. 100 vs 100, shown by the symbol ■) against which the other strains have been normalised.
4.2.2.3 β1,4-GalTase mRNA levels in other tissues from MRL lpr/lpr mice

The mRNA level of β1,4-GalTase was measured in non-lymphoid tissues (liver and kidney) and in spleen from MRL lpr/lpr mice and compared to those in CBA/Ca mice. Pools of two mice from each strain were used in this study. Spleen organs from individual mice were divided into two. Spleen suspensions were made from half of the tissues and splenic lymphocytes were selected after plastic adherence. The other halves of the spleens were homogenised (as a tissue) to isolate RNA for further analysis.

As seen in Figure 4.9, kidney and liver tissues from MRL lpr/lpr and CBA/Ca mice contain similar levels of β1,4-GalTase mRNA. However, the β1,4-GalTase mRNA level is reduced in spleen tissue and shows even further reduction in splenic lymphocytes of MRL lpr/lpr mice compared to those of CBA/Ca mice.
4.2.2.4 β1,4-GalTase gene expression in B cells from MRL lpr/lpr mice

The arthritis-prone MRL lpr/lpr strain mice, the congenic strain MRL +/+ and the CBA/Ca strain with normally galactosylated IgG, all showed similar levels of β1,4-GalTase mRNA in surface-IgG positive splenic B cells. However, β1,4-GalTase mRNA levels were reduced in surface-IgG negative non-adherent spleen cells from both MRL lpr/lpr and MRL +/+ strain mice compared to CBA/Ca mice (Figure 4.10). In the lymph node compartment, β1,4-GalTase mRNA levels were again comparable in surface-IgG positive cells from all three strains of mice. For the surface-IgG negative non-adherent lymph node cells, the mRNA levels for this enzyme were much reduced in MRL lpr/lpr mice compared to that in both MRL +/+ and CBA/Ca strain mice (Figure 4.9). The average of % G0 levels for the serum IgG obtained from CBA/Ca, MRL +/+, and MRL lpr/lpr mice used in this study were 26.3, 32.1, and 49.9 respectively (% G0 was measured by K. Bodman).

Figure 4.10  Quantitation of the β1,4-GalTase mRNA levels for B cells (IgG⁺) and IgG-negative non-adherent cells (IgG⁻) from spleen and lymph nodes of MRL lpr/lpr, MRL +/+, and CBA/Ca strain mice (arbitrarily assigned a value of 100 for each experiment). Error bars indicate ± standard error of the mean for three separate RPA measurements on each sample. Each sample contains total RNA from pools of seven mice.
4.2.2.5 Distribution of G0 secreting B-cells in arthritis-prone versus control mice

This section was done in collaboration with K. Bodman. In MRL/lpr/lpr mice the G0 values of the secreted IgG in peripheral blood and spleen cell cultures were significantly higher when compared to bone marrow (p=0.007 and p=0.004 respectively, Figure 4.11) but not lymph node (p>0.2). We also measured the concentration of total IgG secreted spontaneously in cell cultures from 4 different lymphoid compartments. The only significant difference was the increased level of secretion by the spleen cell suspensions compared to the bone marrow (p=0.024, Figure 4.11).

In the control strain CBA/Ca, the amount of IgG secreted and the percentage IgG G0 were not significantly different in any of the compartments studied.

When comparing the two strains, G0 levels of secreted IgG from peripheral blood and spleen cell cultures (but not from bone marrow or lymph node) were significantly higher in MRL/lpr/lpr mice (p=0.041 and p=0.006 respectively) reflecting the increased serum IgG G0 levels in these mice (p<0.001). The production of IgG (IgG concentration) was raised significantly only in the MRL/lpr/lpr splenic cell supernatants when compared to CBA/Ca (p=0.03).
Figure 4.11. G0 levels (top) and IgG concentrations (bottom) for de novo secreted IgG from spleen, lymph node, bone marrow, and peripheral blood, and for serum (G0 only), with mean ± standard error of the mean from MRL lpr/lpr (solid circle) and CBA/Ca (open circle) mice. This experiment was performed by K. Bodman.
4.2.3 Studies on cell lines

The levels of β1,4-GalTase mRNA for three EBV-transformed B-lymphoblastoid cell lines (BRAD-3, JAC10, and 2B6) cultured at four different cell densities are shown in Figure 4.12. The data represents the mean for three separate experiments. No statistically significant difference is observed between the β1,4-GalTase mRNA levels at different cell densities from each cell line nor between the β1,4-GalTase mRNA levels at the same cell density from different cell lines. However, the average of β1,4-GalTase mRNA levels for all four cell densities for the cell line BRAD-3 (β1,4-GalTase/18S rRNA=0.49) is 40% lower than that of the JAC10 cell line (0.68). The average of β1,4-GalTase mRNA levels measured for the cell line 2B6 (0.65) for the four cell densities is comparable to that of JAC10 cell line (Table 4.2).

![Figure 4.12](image)

**Figure 4.12** Comparison of the mRNA levels for β1,4-GalTase in three different EBV-transformed B-lymphoblastoid cell lines (BRAD-3, JAC10, and 2B6) cultured at four different cell concentrations. The error bars indicate mean of three separate experiments ± standard error of the mean. For comparison purposes, results obtained in each experiment are normalised against the mean mRNA level obtained for the same human PBL sample (arbitrarily assigned a value of 1.0) used in all experiments.
The levels of digalactosyl, monogalactosyl, and agalactosyl IgG secreted from the three cell lines, together with the levels of β1,4-GalTase enzyme activity (Kumpel et al. 1994) and mRNA in the lymphoblastoid cells are provided in Table 4.2.

**Table 4.2** Comparison of IgG oligosaccharide galactosylation, β1,4-GalTase enzyme activity and gene expression in three EBV-transformed B-lymphoblastoid cell lines. The data in the shaded area are taken from Kumpel et al. 1994.

<table>
<thead>
<tr>
<th>Monoclonal anti-D</th>
<th>2B6</th>
<th>JAC10</th>
<th>BRAD-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>% G0</td>
<td>3.6</td>
<td>4.5</td>
<td>2.2</td>
</tr>
<tr>
<td>% G1</td>
<td>17.7</td>
<td>24.4</td>
<td>20.9</td>
</tr>
<tr>
<td>% G2</td>
<td>78.7</td>
<td>71.1</td>
<td>76.9</td>
</tr>
<tr>
<td>β1,4-GalTase enzyme activity (pmol/min/mg)</td>
<td>131 ± 12</td>
<td>109 ± 11</td>
<td>51 ± 7</td>
</tr>
<tr>
<td>β1,4-GalTase mRNA : 18S rRNA</td>
<td>0.65 ± 0.16</td>
<td>0.68 ± 0.30</td>
<td>0.49 ± 0.15</td>
</tr>
</tbody>
</table>
4.3 DISCUSSION

It is shown here that peripheral blood and spleen cell cultures from MRL /pr/lpr mice secrete agalactosylated IgG in vitro. Previously, it had been shown that immunoglobulins newly-secreted from PWM-stimulated lymphocytes from RA patients had reduced levels of galactose (Bodman et al 1992). These data imply that the defect in IgG galactosylation is at least in part attributable to the biosynthetic pathways. As discussed earlier, galactose is added to the oligosaccharide chains of IgG by the enzyme β1,4-GalTase in trans-cisternae of the Golgi complex. The glycosylation process is tightly regulated, although not much is known about the control mechanisms. Part of this control comes from the specificity and the amount of glycosyltransferases present (cell and tissue specific glycosylation), the primary peptide structure, and the constraints imposed by the three-dimensional structure of individual proteins (Dwek 1995).

The defect in galactosylation in RA seems to be restricted to IgG. Transferrin (Furukawa et al 1990), IgA (Field et al 1994), IgM (Rademacher et al 1996), and the Fab of IgG (Parekh et al 1985, Dwek 1995) have almost fully galactosylated oligosaccharides in patients with RA. Galactosylation at the Fc site of IgG is complicated by the unique structure of IgG in that unlike other glycoproteins that have their carbohydrates exposed, the IgG oligosaccharide moiety is contained within the space between the Fc Cγ2 polypeptide domains. Therefore, it could be that galactosylation at this site is more sensitive to small fluctuations in the level of enzyme β1,4-GalTase, or alternatively the enzyme level is not the limiting factor but that the rate of H-H chain disulphide bond formation in the Golgi determines the efficiency of galactosylation at this site (Rademacher et al 1995).

Although glycosyltransferases, in general, are thought to be regulated at the transcriptional level, there is some evidence for post-translational regulation
of the β1,4-GalTase enzyme. This enzyme has been shown to be phosphorylated at serine residues (Strous et al. 1987) and there is some evidence that the p58GTP protein kinase may be involved in the regulation of β1,4-GalTase activity through phosphorylation (Bunnell et al. 1990).

Data on β1,4-GalTase enzyme activity (Axford et al. 1987, Furukawa et al. 1990, Wilson et al. 1993, Axford 1992, Keusch et al. 1995) suggest that the glycoprotein acceptor and/or the method of B cell preparation used in each study contribute to the observed activity of the β1,4-GalTase enzyme. The observation of similar levels of β1,4-GalTase mRNA in peripheral CD19+ B cells from patients with RA and normal controls, and also in both splenic and lymph node slgG+ B-cells from arthritis-prone and control mice suggest that the B-cell β1,4-GalTase activity, if reduced in RA, must be post-transcriptionally regulated.

The mRNA levels for the enzyme have been measured in circulating peripheral blood B-cells, the majority of which are resting virgin and memory cells. This might not reflect the levels of the message in the plasma cells which are the source of the defectively glycosylated serum IgG. It is not possible to directly isolate sufficient numbers of plasma cells to provide enough RNA for the RPA measurements, and the use of in vitro techniques such as EBV transformation has been shown to increase β1,4-GalTase activity in RA B-cells leading to the conclusion that such procedures can not be used in these types of study (Wilson et al. 1993). An alternative approach might be to use RT-PCR but such assays are accurately described as "semi-quantitative" and are unlikely to provide as accurate a quantitation of mRNA levels as the RPA. All previous studies on β1,4-GalTase activity in RA have examined B-lymphocytes rather than plasma cells and the aim of the present study was to establish if the reported decrease in β1,4-GalTase activity in this cell type in RA can be explained by a decreased production (or alternatively increased turn-over) of the specific mRNA species. These studies clearly show this not to be the case.
In the murine studies, initially, the primary aim was to measure mRNA levels accurately in individual mice. To obtain enough material to do this, it was not possible to separate T and B cells or measure β1,4-GalTase activity in the samples from individual mice. The results showed that there were reduced levels of β1,4-GalTase mRNA in splenic lymphocytes (spleen cell suspension depleted of plastic-adherent cells) from MRL /pr//pr mice. The observation of similar levels of β1,4-GalTase mRNA in kidney and liver tissues from MRL /pr//pr and CBA/Ca mice suggested that the reduction in the β1,4-GalTase mRNA may be confined to the lymphoid organs of the MRL /pr//pr mouse. To investigate further, separated IgG-positive and IgG-negative (cell suspension depleted of plastic-adherent cells and slgG⁺ cells) cells from spleen and lymph nodes from pools of mice were examined for their level of β1,4-GalTase mRNA. Reduced levels of β1,4-GalTase mRNA observed earlier in splenic lymphocytes were reflected in the decreased levels of the message in the IgG-negative populations from both spleen and lymph nodes, suggesting a further abnormality in MRL /pr//pr T cells. These findings would correspond with those of Imai and colleagues (Imai et al 1988) who report reduced binding to MRL /pr//pr lymph node T cells of the lectins RCA and allo A which bind primarily to a Galβ1-4GlcNAc structure. Contrary to this observation, the same authors reported an increase in the activity of β1,4-GalTase in MRL /pr//pr lymph node T cells using asialo-agalacto-transferrin as the acceptor (Imai et al 1988).

There is also evidence for defective glycosylation of other molecules, such as CD45 (Yamashita et al 1989), Forssman and Paul-Bunnel antigens (Katagiri et al 1984), and possibly G₃,₄ gangliosides (Ishii and Watanabe 1992) in MRL /pr//pr lymph node cells. Whether the glycosylation abnormalities observed in MRL /pr//pr mice are associated with the defect in fas-mediated apoptosis in B220⁺, CD4⁻, CD8⁻ (DN) T cells (Watanabe-Fukunaga et al 1992) remains to be established. These DN T cells have been found to show many abnormalities in TCR/CD3 initiated signal transduction pathways, resulting in a severe deficiency in proliferative responses (Altman 1994). In fact, MRL
*lpr/lpr* DN T cells bear many similarities to normal anergic T cells, and both cell types can be rescued from this tolerant condition through the induction of cell cycling in the presence of IL-2 (Beverly et al. 1991). On the other hand, there is evidence that \( \beta \)-1,4-GalTase gene expression is regulated during entry into the cell cycle and during the cell cycle, with the lowest level in quiescent (G0 stage of the cell cycle) cells and increasing levels upon restimulation of cell growth (Pouncey et al. 1991; Masibay et al. 1991). Therefore, it is possible that the reduction in \( \beta \)-1,4-GalTase mRNA we observe in the IgG-negative population may be a consequence of the anergic state of DN T cells in MRL *lpr/lpr* mice.

In contrast to the IgG-negative lymphocytic population, IgG-positive B cells from both spleen and lymph nodes of MRL *lpr/lpr* showed mRNA levels comparable to those of CBA/Ca and MRL +/-+. This supports the observations of Axford and coworkers (Axford et al. 1994) who have reported a reduction in peripheral but not splenic B lymphocyte \( \beta \)1,4-GalTase activity in MRL *lpr/lpr* mice compared to CBA/Ca mice, and no significant difference in B cell GalTase activity when comparing MRL *lpr/lpr* with MRL +/-+.

Studies carried out using the IgG-secreting cell lines showed that the BRAD-3 cell line with the lowest \( \beta \)1,4-GalTase enzyme activity (Kumpel et al. 1994) also exhibited, on average, the lowest level of \( \beta \)1,4-GalTase mRNA. The two other cell lines showed comparable mRNA and enzyme activity levels, although they did not correlate exactly. The variation in mRNA levels determined for the three experiments is greater than expected, especially for the JAC10 cell line. The reason for this is not clear. The results of the present study also show that changes in cell density (10-fold variation) does not significantly change the expression of the \( \beta \)1,4-GalTase gene in the three cell lines examined.

The lower mRNA and enzyme activity levels in the BRAD-3 cell line does not correlate with the highly galactosylated IgG level in this cell line. In addition,
the β1,4-GalTase mRNA levels of these EBV-transformed cell lines are not greater than that of a sample isolated from peripheral blood lymphocytes of a normal individual (which is used as an inter-experimental control in the study on cell lines). The levels of β1,4-GalTase enzyme activity in the EBV-transformed cell lines were also shown to be within or close to the range obtained for Ficoll prepared fresh B lymphocytes (55-161 pmol/min/mg) and EBV-transformed B-cell lines from patients with RA and controls (56-273 pmol/min/mg) (Wilson et al 1993). Taken together, these data would indicate that B-cells producing highly galactosylated anti-D IgG molecules do not require increased levels of β1,4-GalTase mRNA and enzyme activity.

Overall studies presented in this chapter indicate that there is no association between β1,4-GalTase mRNA levels and IgG galactosylation in RA B-cells. Evidence obtained using lymphoblastoid cell lines also points to a lack of correlation between β1,4-GalTase mRNA (present study) and enzyme activity levels (Kumpel et al 1994) and the extent of galactosylation. It may be that β1,4-GalTase enzyme levels are not directly related to the galactosylation defect of IgG, and that some other abnormality in IgG biosynthesis or regulation of glycosylation may be causing the defective IgG galactosylation seen in RA.
CHAPTER 5

IgG GLYCOSYLATION AND $\beta_{1,4}$-GALTASE DURING PREGNANCY AND LACTATION
5.1 INTRODUCTION

Patients with RA often show improvement or remission during pregnancy and exacerbation of the disease during the post-partum period (Hench 1938, Oka and Vainio 1966, Cecere and Persellin 1981, Ostensson and Husby 1983). Similar changes have been observed in several animal models of the human disease including murine collagen II-induced arthritis (Waites and Whyte 1987), Pristane-induced arthritis (Thompson et al 1992), proteoglycan-induced arthritis (Buzas et al 1993) and the spontaneous arthritis associated with MRL lpr/lpr (Ratkay et al 1994). Although hormonal changes have been suggested to play an important role as the underlying biochemical alteration, the factor(s) of prime aetiologic significance which is produced in pregnancy, has not yet been identified. Changes in IgG galactosylation are reported to be temporally associated with remission of arthritis during pregnancy. The percentage of galactosylated IgG rises during normal human and murine pregnancy and falls to values lower than before conception following delivery. The changes in IgG glycosylation in pregnant arthritic women and mice occur simultaneously with the pregnancy-induced remission and post-partum recurrence of disease (Pekelharing et al 1988, Rook et al 1991b, Thompson et al 1992).

It has been speculated that the fluctuations in IgG galactosylation observed in pregnancy might reflect changes in the β1,4-GalTase enzyme levels (Rook et al 1991b, Thompson et al 1992). In pregnancy, as discussed earlier, the specificity of β1,4-GalTase in lactating mammary gland is altered by binding to a modifier subunit, α-lactalbumin, to catalyse the synthesis of lactose. The expression of the β1,4-GalTase gene in mammary glands isolated from mid- to late pregnant and lactating mice is ~10-fold higher relative to somatic tissue (Harduin-Lepers et al 1993). There is also evidence that other glycosyltransferases involved in the biosynthesis of asparagine-linked oligosaccharides on glycoproteins are regulated in the mammary glands.
during the gestational period and into lactation (Vijay et al 1986, Lacord-Bonneau et al 1988).

To investigate whether the changes in IgG galactosylation that are seen in pregnancy are also accompanied by alterations in lymphocytic β1,4-GalTase enzyme, we have measured the level of β1,4-GalTase gene expression and enzyme activity in spleen cells from pregnant and lactating mice in relation to the galactose content of their serum IgG.

Changes in the level of prolactin during pregnancy and breast-feeding has been suggested as a cause for improvement of RA during pregnancy and flare-up of the disease post-partum (Markoff et al 1988, Berczi 1993, Brennan and Silman 1994). In addition, prolactin has been reported to increase the enzyme activity of β1,4-GalTase in the rat mammary gland (Ip and Dao 1978) and also the enzyme activity and mRNA accumulation of β1,4-GalTase in the mouse mammary gland (Golden and Rimella 1995, Jagoda and Rimella 1991, Turkington et al 1968). The intra-peritoneal injection of prolactin into male monkeys, however, has been shown to decrease the enzyme activity of β1,4-GalTase in the epididymal tissue (Jayakumar et al 1992).

The effect of prolactin on β1,4-GalTase gene expression in B cells has not previously been investigated. Prolactin receptors are present on B cells (Russel et al 1984, Dardenne et al 1994) and prolactin has been shown to have modulatory effects on the B-cell function. Hypophysectomised animals have depressed humoral and cell-mediated immune function which can be restored after reconstitution of normal prolactin levels (Reber 1993). In addition, in vitro studies have shown that physiological concentrations of prolactin (12-25 ng/ml) increases the response of B cells to mitogenic stimuli and concentrations of prolactin 5-10-fold the physiological levels inhibits this mitogenic response (Matera et al 1992). It appears that an optimal amount of prolactin must be present for maximal immune function and either too much or too little prolactin may be immunosuppressive (Reber 1993). In the present
study, we have analysed the direct effect of prolactin on IgG galactosylation and β1,4-GalTase gene expression in vitro using human peripheral blood lymphocytes. Initially, the effect of prolactin in combination with insulin and hydrocortisone was studied on IgG galactosylation and the expression of the β1,4-GalTase gene in human peripheral blood lymphocytes. This combination of hormones was shown to increase the mRNA and activity of β1,4-GalTase in the murine mammary gland explants (Golden and Rimella 1995, Jagoda and Rimella 1991, Turkington et al 1968). The effect of prolactin was also studied on enriched human peripheral blood B-cells (to remove any possible diluting effect from the β1,4-GalTase levels in T cells) activated by anti-IgM antibodies and IL-2.
5.2 RESULTS

5.2.1 The effect of pregnancy and lactation on murine β1,4-GalTase and IgG glycosylation

5.2.1.1 Experimental design
Six female mice were left as controls and the others were mated with normal Balb/c males. The day of finding a vaginal (copulation) plug was designated the first day (day 1) of pregnancy. The females were separated from the males after detecting the vaginal plug and housed one per cage. Five mice were culled on day 8 and five mice on day 15 of pregnancy. Six mice were left to go through parturition and the litters were not weaned from the mothers until the termination of the studies, on day 8 postpartum. After sacrificing mice by CO\textsubscript{2} inhalation, blood was taken by cardiac puncture, spleens were removed into ice-cold medium and mammary glands were removed, snap-frozen in liquid nitrogen and kept at -70°C until further analysis. The mating was planned so that the average age of mice in each group was similar (16.5 ± 0.3 weeks) at the termination of study. Pregnancy in 8-days and 15-days pregnant mice was confirmed by observing the foetuses in the uteri.

5.2.1.2 IgG concentration
The total IgG concentration was found to be reduced five fold in 15-days pregnant (p<0.001, unpaired Student's t-test) and lactating (p<0.001) mice compared to the age-matched non-pregnant mice (Figure 5.1). Eight-days pregnant mice showed levels of serum IgG that were not significantly different (p=0.84) from those found in the control non-pregnant mice.
Figure 5.1  Serum IgG concentration in non-pregnant, 8- and 15-day pregnant, and lactating 8-day post-partum mice. Mean ± standard error of the mean is indicated (5 mice for 8-days and 15-days pregnant, 6 mice for non-pregnant and post-partum).

5.2.1.3 IgG galactosylation

The level of IgG galactosylation, as measured by the ratio of RCAI : BSII binding, increased in 15-days pregnant (p<0.001) and lactating (p<0.001) mice compared to the age-matched non-pregnant mice (Figure 5.2). Eight-days pregnant mice showed IgG galactosylation that was not significantly different (p=0.34) from that found in the control non-pregnant mice. To exclude the possibility that the decreased levels of IgG in the pregnancy and post-partum sera would influence the result, the ELISA plates were coated with a concentration of Protein G' which would be fully saturated with IgG even when using those sera in which the lowest levels of IgG were found.
Figure 5.2 Level of agalactosyl IgG in non-pregnant, 8- and 15-day pregnant, and lactating 8-day post-partum mice as determined by the ratio of BSII binding to GlcNAc over RCAI binding to galactose. Mean ± standard error of the mean is indicated (5 mice for 8-days and 15-days pregnant, 6 mice for non-pregnant and post-partum).

5.2.1.4 β1,4-GalTase enzyme activity

The level of β1,4-GalTase enzyme activity (measured by J. Keusch) did not change significantly in spleen cells during the gestation period and postpartum compared to the enzyme activity in control non-pregnant mice (Figure 5.3).
5.2.1.5 β1,4-GalTase Gene Expression

Spleen cells from non-pregnant, 8- and 15-day pregnant, and lactating mice had comparable levels of β1,4-GalTase mRNA. The level of β1,4-GalTase mRNA in mammary tissues, however, was increased significantly in the 15-days pregnant mice compared with 8-days pregnant and was even higher in the lactating mice (Figure 5.4 and 5.5).
Figure 5.4a  RNase protection analysis of splenic β1,4-GalTase RNA in non-pregnant (lane 9), 8- and 15-day pregnant (lanes 10 and 11), and lactating 8-day post-partum (lane 12) mice, and of mammary tissue β1,4-GalTase from post-partum (lane 13) and non-pregnant (lane 14) mice.

Controls: Lane 1, yeast tRNA hybridised with β1,4-GalTase and GAPDH probes and treated with RNase. Lane 2, molecular size marker (HaeIII restriction fragments of PhiX174 DNA). Lane 3, yeast tRNA hybridised with β1,4-GalTase probe alone but without RNase, showing full length 602-nt β1,4-GalTase probe. Lane 4, yeast tRNA hybridised with GAPDH probe alone but without RNase, showing full length 404-nt GAPDH probe. Lane 5, murine spleen RNA hybridised with β1,4-GalTase probe alone to show 559-nt protected fragment. Lane 6, murine spleen RNA hybridised with GAPDH probe alone to show 316-nt protected fragment. Lanes 7 and 8, double and triple amount of RNA from lane 10 hybridised with both β1,4-GalTase and GAPDH, showing the probes are in molar excess over the target mRNA.
**Figure 5.4b** β1,4-GalTase mRNA levels assessed by RNase protection analysis of total RNA isolated from mammary tissues from non-pregnant (lanes 4 and 5), 8-day (lanes 6 and 7) and 15-day (lanes 8 and 9) pregnant, and lactating 8-day post-partum (lanes 10 and 11) mice. Results for two individual mice are shown.

*Controls:* Lane 1, molecular size marker. Lane 2, RNA from mammary tissue hybridised with β-actin probe showing 250-nt protected fragment. Lane 3, RNA from mammary tissue hybridised with β1,4-GalTase showing 559-nt protected fragment. Lane 12, yeast tRNA hybridised with β1,4-GalTase and β-actin probes and treated with RNase. Lane 13, yeast tRNA hybridised with β1,4-GalTase in the absence of RNase showing full length 602-nt β1,4-GalTase probe. Lane 14, yeast tRNA hybridised with β-actin in the absence of RNase showing full length 304-nt β-actin probe.
Figure 5.5a β1,4-GalTase mRNA levels in spleen cells using RPA. Results are expressed as the ratio of β1,4-GalTase over GAPDH mRNA signal. Mean ± standard error of the mean is indicated (5 mice for 8-days and 15-days pregnant, 6 mice for non-pregnant and post-partum).

Figure 5.5b β1,4-GalTase mRNA levels in total RNA isolated from mammary tissues of mice at different stages of gestation. Results are expressed as the ratio of β1,4-GalTase over β-actin mRNA signal. Mean ± standard error of the mean is indicated (5 mice for 8-days and 15-days pregnant, 6 mice for non-pregnant and post-partum).
5.2.2 The effect of prolactin / insulin / hydrocortisone on IgG glycosylation and β1,4-GalTase mRNA in human PBL

β1,4-GalTase mRNA levels were measured after stimulation of peripheral blood lymphocytes with prolactin, insulin, and hydrocortisone (Hc). Two experiments were performed. In experiment “a” the cells were stimulated for 0.5, 1.0, 2.0 and 3.0 hours and in experiment “b” for 15 and 40 hours. The control cells consisted of peripheral blood lymphocytes in medium only. Similar levels of β1,4-GalTase mRNA were observed in the control and test samples in the specified time period. Comparable levels of IgG galactosylation were observed (measured by K. Bodman) for de novo secreted IgG from the cells cultured for 7 days in the presence of prolactin, insulin, and hydrocortisone (22.3% G0) and in medium only (21.3% G0).

![Graph](image-url)

**Figure 5.6** Quantitation of the β1,4-GalTase mRNA levels for human peripheral blood lymphocytes stimulated with prolactin, insulin, and hydrocortisone (Hc) for specified time points. These results were obtained from two separate experiments (a and b) using lymphocytes from two individuals, and therefore the two experiments are not directly comparable. Error bars indicate ± standard error of the mean for duplicate RPA measurements.

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5.2.3 The effect of prolactin on human peripheral blood B-cell β1,4-GalTase mRNA and IgG glycosylation

The galactosylation level of secreted IgG and the level of β1,4-GalTase mRNA was measured with respect to the effect of prolactin on activated peripheral blood B-cells (i.e. in the presence of anti-IgM antibody and IL-2). Two experiments were carried out. In experiment 'a', the control sample consisted of peripheral blood B cells in medium only (i.e. not activated). In experiment 'b', the control sample consisted of the peripheral blood B cells which were activated by the addition of anti-IgM antibody and IL-2.

5.2.3.1 β1,4-GalTase gene expression

Prolactin increased the mRNA levels for β1,4-GalTase in enriched peripheral blood B lymphocytes (non-plastic-adherent, SRBC rosette-depleted) activated with anti-IgM antibodies and recombinant IL-2 by 34% (1.3-fold) compared to cells in medium only (Figure 5.7a) and 26% (1.3-fold) compared to cells activated with anti-IgM antibody and IL-2 (Figure 5.7b).

5.2.3.2 IgG concentration and galactosylation

The total IgG concentration secreted by the enriched peripheral blood B-cells (cultured for 7 days) in the presence of prolactin, anti-IgM antibodies and IL-2 was 2.7-fold higher than the IgG secreted by cells cultured with anti-IgM antibodies and IL-2 only and 4.6-fold higher than the spontaneously secreted IgG by cells cultured in medium only. No changes in the number of terminal galactose residues of IgG-associated oligosaccharides were found upon the addition of prolactin to enriched peripheral blood B-cells in culture (Table 5.1).
Figure 5.7 The expression of β1,4-GalTase gene in enriched peripheral blood B-cells in the presence of prolactin, anti-IgM antibodies and IL-2. The control sample represents cells cultured with either medium only (a) or with anti-IgM antibodies and IL-2 (b). The results, obtained by phosphoimage analysis of signals for β1,4-GalTase mRNA and 18S rRNA and expressed as a ratio. Error bars indicate duplicate RPA measurements on the same sample.

Table 5.1 Total IgG concentration secreted by enriched peripheral blood B-cells in culture (for 7 days) and galactosylation status of secreted IgG as measured by the BSII / RCAI binding. The data in experiment “a” were obtained by K. Bodman.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cells +</th>
<th>IgG Concentration</th>
<th>BSII / RCAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>anti-IgM Ab + IL-2 + prolactin medium only</td>
<td>0.23 µg/ml</td>
<td>0.349 (17.0% G0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05 µg/ml</td>
<td>0.378 (17.4% G0)</td>
</tr>
<tr>
<td>b</td>
<td>anti-IgM Ab + IL-2 + prolactin anti-IgM Ab + IL-2</td>
<td>0.19 µg/ml</td>
<td>0.585</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.07 µg/ml</td>
<td>0.581</td>
</tr>
</tbody>
</table>
5.3 DISCUSSION

In this study, alterations in the glycosylation of murine IgG which occurs during the normal course of pregnancy have been described. Specifically, increased levels of terminal galactose were observed on serum IgG from late (day 15), but not early (day 8), pregnant Balb/c mice. Rook and colleagues (1991b) detected a similar change in IgG galactose in DBA/1 mice, but in this strain the changes were detected as early as the fourth day of pregnancy. Studies in CBA/Ig<sup>h</sup> strain mice (Thompson et al 1992) and in human subjects (Rook et al 1991b, Pekelharing et al 1988) have also shown increased IgG galactose during pregnancy. In the present study the level of IgG galactosylation in the Balb/c mice was shown to be still raised 8 days post-partum in lactating mice.

Fifteen day pregnant and lactating Balb/c mice were shown to have serum IgG levels that were reduced by approximately 80% compared to the non-pregnant mice. Similarly striking decreases in IgG levels are seen in CBA/Ca (71% reduction at 16 days of pregnancy; Sulila and Mattsson 1990) and C3H/He (approximately 70% reduction at 12 days of pregnancy; Yokoyama et al 1988) mice. Our results show that the decrease in IgG levels in Balb/c mice is temporally related to the increase in IgG galactose levels, i.e. it was seen in late pregnancy and post-partum, but not during early pregnancy. Several factors have been proposed as contributing toward the dramatic decrease in IgG concentration during murine pregnancy, including the pregnancy associated increased plasma volume (Sulila and Mattsson 1990, Ailus 1994), selective transport of IgG to the fetus (Sulila and Mattsson 1990), increased IgG degradation (Sulila and Mattsson 1990), selective B-cell clonal inactivation (Yokoyama et al 1988) and T-cell mediated suppression of immunoglobulin class switching (Yokoyama et al 1988). B-cell development has been found to be suppressed during pregnancy (Medina et al 1993) and lactation is known to delay the regeneration of B-cell precursors in the bone marrow of post-partum mice (Medina et al 1993). Serum IgG1 and IgG2 levels have also been shown to be reduced in lactating mice (Mink and Benner...
It is possible that a decreased rate of production of IgG might permit a longer trans-Golgi transit time, with a concomitant increase in the percentage of immunoglobulin heavy chains which become galactosylated.

The addition of galactose to terminal GlcNAc on the IgG oligosaccharide is dependent on the presence of β1,4-GalTase. We found that during pregnancy expression of β1,4-GalTase mRNA in the spleen, a major source of circulating IgG and therefore agalactosyl IgG, did not increase as determined using an extremely sensitive RPA technique, an approach which readily detected the known pregnancy-associated increase in mammary gland β1,4-GalTase mRNA (Harduin-Lepers et al. 1993). Furthermore, the enzyme activity in the spleen remained relatively constant throughout pregnancy.

In these experiments, equal amounts of input RNA were used as the target for each tube of the RPA. This was based on measurement of the absorbance of the RNA preparation at 260 nm. Additionally, a labelled probe for a house-keeping gene was included in each experiment. This was GAPDH in the case of splenic tissue, but β-actin for mammary tissue because expression levels of GAPDH had already been reported to fluctuate in mammary tissue (Spanakis 1993). Although it was hoped that these would act as reliable internal standards to confirm that equal amounts of input RNA had indeed been used, differences in the intensity of the bands corresponding to the message from these two genes were seen. The reproducible findings in this study suggest that neither of these genes are always expressed at a stable level, a conclusion which has been stated by others as well (Bhatia et al. 1994, Spanakis 1993, Finnegan et al. 1993, Hoang-Vu et al. 1992).

In the murine studies reported in this chapter, β1,4-GalTase mRNA levels were measured in splenic lymphocytes rather than in purified B-cells. There is no evidence for B-cell specific regulatory elements within the β1,4-GalTase gene locus comparable to the mammary cell specific promoter.
Lepers et al. (1993) which may be responsive to hormones such as prolactin. Prolactin is a pro-inflammatory hormone which is also necessary for lactation. During pregnancy, prolactin levels increase along with levels of anti-inflammatory steroidal hormones. After parturition the levels of all these hormones will fall rapidly to their pre-pregnancy levels unless the mother is breast-feeding, in which case prolactin levels will remain high. Exposure to breast-feeding has been found to be associated with a significant increase in risk of developing RA (Brennan and Silman, 1994). Moreover, investigations on the role of prolactin in RA is not confined to pregnancy. Prolactin has also received considerable attention as one of the hormones involved in the pathogenesis of RA. The most recent study on the levels of prolactin in patients with active RA reported a normal diurnal pattern of secretion but at a significantly elevated level at all time points compared with patients with active osteomyelitis (Chikanza et al., 1993). The same authors also found an abnormal increase in plasma prolactin concentration following surgery in patients with RA compared to patients with osteoarthritis and osteomyelitis.

Other studies have found normal levels of prolactin in patients with RA (Hedman et al., 1992, Berczi et al., 1987). Although in the study carried out by Berczi and colleagues prolactin levels were reported to be normal in patients with RA, a decrease in the bioactivity of circulating prolactin (as determined by measuring the proliferation of Nb2 rat lymphoma cell line which is dependent on prolactin for growth) was reported (Berczi et al., 1987, Nagy et al., 1991). The differences observed in these studies may be explained by the diurnal pattern of prolactin secretion. Inconsistency in the timing of blood collection from patients and normal subjects may obscure the results. In fact, prolactin secretion was found to be maximum during the night, the time when cortisol secretion was at the lowest level. It is suggested that the combination of low cortisol, which has anti-inflammatory properties, and high prolactin concentrations may result in the worsening of RA disease activity at night (Chikanza et al., 1993). Increased serum prolactin levels have also been found to be associated with the disease activity in patients with RA in a study by Halko et al. (1995).
Rillema and colleagues have shown that prolactin enhances the activity of and mRNA accumulation for, β1,4-GalTase in mammary tissue (Jagoda and Rillema 1991, Golden and Rillema 1995). Although B-cells also express receptors for prolactin (Dardenne et al. 1994, Russel et al. 1984) we have found no effect on β1,4-GalTase mRNA levels when human PBL were cultured in the presence of prolactin, insulin and hydrocortisone; a combination which has been shown to increase β1,4-GalTase mRNA levels in mammary tissue (Jagoda and Rillema 1991, Golden and Rillema 1995). However, when prolactin, in combination with the cellular activators anti-IgM and interleukin-2 which cause increased IgG secretion by B-cells (Lahat et al. 1993), was added to B-cell enriched (non-plastic-adherent, SRBC rosette-depleted) peripheral blood mononuclear cells, there was a small increase in β1,4-GalTase mRNA compared to the levels without prolactin, but there was no effect on IgG galactosylation.

In general, protein glycosylation is thought to be controlled by alterations in the activity of glycosyltransferases and that the amount of these enzymes is mainly regulated at the level of gene transcription, with enzyme activity directly correlating with the level of mRNA (Kleene and Berger 1993). The present study shows for the first time, that the physiological pregnancy-associated alteration in IgG glycosylation is not due to this type of regulation. Thus, protein structure effects related to the internal nature of the Fc-associated oligosaccharide (Rademacher et al. 1994b) are likely to play a key role in the control of IgG glycosylation.
CHAPTER 6

CYTOKINE AND T-CELL CONTROL OF β1,4-GALTASE GENE EXPRESSION
6.1 INTRODUCTION

There is now considerable evidence to suggest that cytokines contribute to the pathogenesis of inflammatory autoimmune diseases such as RA, either directly by causing tissue destruction or indirectly through the activation of autoreactive and inflammatory cells. There is a consensus that interleukin 1 (IL-1), interleukin 6 (IL-6), granulocyte/macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor (TNF-α) are present at high concentrations within the joint in patients with RA (Buchan et al. 1988, Firestein et al. 1990). Other cytokines that have been detected in the RA synovium in abundance include IL-8, IL-10, and TGF-β (Brennan et al. 1990a, Brennan et al. 1990b, Katsikis et al. 1993). T cell derived cytokines such as IL-2 and gamma-interferon (IFN-γ) are produced in less abundance (Buchan et al. 1988).

6.1.1 IL-6

Amongst the many cytokines present in the RA synovium, IL-6 is of particular interest with respect to hypogalactosylation of IgG. IL-6 is known to belong to a family of glycosylation regulating cytokines (Mackiewicz et al. 1989b) and there is evidence that IL-6 can regulate the glycosylation of acute phase proteins (Pos et al. 1989, Mackiewicz and Kushner 1989). In patients with Castleman’s disease, hypogalactosylation of IgG has been linked with raised levels of IL-6 (Nakao et al. 1991), which is produced by activated B cells in the germinal center of hyperplastic lymph nodes. Both a clinical improvement and a decrease in serum IL-6 levels were observed after removing these lymph nodes (Yoshizaki et al. 1989). An association between raised serum agalactosyl IgG and increased IL-6 levels has also been reported in patients with sarcoidosis (O’Conner et al. 1992), cardiac myxoma (Nakao et al. 1991) or myelomas (Nishiura et al. 1990).

In addition, there is evidence that IL-6 might be linked with the elevated levels of agalactosyl IgG found in patients with RA. Higher levels of IL-6 are
detected in the sera from patients with RA compared to controls (Holt et al 1992, Houssiau et al 1988). Levels of IL-6 have also been found to be further increased in synovial fluid compared to serum in patients with RA (Hirano et al 1988, Houssiau et al 1988, Bhardwaj et al 1989, Wood et al 1992, Swaak et al 1988, Firestein et al 1988). In another study, however, neither levels of serum nor synovial fluid G0 have been found to correlate with soluble IL-2R or IL-6 (Bond et al 1994). There is a significant correlation between the level of synovial IL-6 and IgG concentration (Hermann et al 1989). Treatment of severe RA by anti-IL-6 monoclonal antibody has been shown to result in significant clinical improvement in a number of different disease activity measures (Wendling et al 1993). Moreover, the level of agalactosyl IgG is reported to be higher in the synovial fluid than in the serum from RA patients (Parekh et al 1989, Tsuchiya et al 1993, Bodman 1995).

There is also evidence linking IL-6 to raised levels of agalactosyl IgG found in the animal models of arthritis. In the MRL lpr/lpr mice, an age-associated increase in both serum IL-6 levels (Tang et al 1991) and agalactosyl IgG levels (Bodman et al 1994) has been reported. IL-6 production is also seen in type II collagen-induced arthritis in mice (Takai et al 1989). Moreover, an association between serum agalactosyl IgG levels and peritoneal exudate fluid (PEF) IL-6 has been demonstrated in pristane-induced arthritis (Hitumoto et al 1992). Thompson et al demonstrated that the kinetics of IL-6 activity post-pristane injection parallels the kinetics of agalactosyl IgG production and the overshoot in agalactosyl IgG levels immediately post-partum coincides with a burst in IL-6 activity (Thompson et al 1992). Injection of recombinant IL-6 into normal mice (CBA/Ig^b) is reported to increase their serum agalactosyl IgG levels (Hitumoto et al 1992) and repeated intraperitoneal injections of IL-6 has resulted in a sustained increase in agalactosyl IgG expression in DBA/1 mice (Rademacher et al 1994).

The most convincing evidence for a link between agalactosyl IgG and IL-6 came from studies on IL-6-transgenic mice which showed strikingly raised
levels of IgG G0 in the serum (Rook et al 1991a). The IL-6 transgenic mice (C57BL/6) were produced using the human IL-6 genomic gene fused with the human immunoglobulin heavy chain enhancer (Eμ). In these IL-6 transgenic mice a massive plasmocytosis was generated in the thymus, lymph nodes and spleen. Infiltration of plasma cells in the lung, liver and kidney was also found. High concentrations of human IL-6 and polyclonal increases in IgG1 were detected in the sera of all transgenic mice (Suematsu et al 1989). These IL-6 transgenic mice developed autoantibodies and mesangial cell proliferative glomerulonephritis, but did not show any sign of arthritis or any other organ-specific autoimmune disease. Another line of IL-6 transgenic mice (H2-IL-6 mice, Woodroofe et al 1992) did not show any sign of arthritis either, suggesting that overexpression of IL-6 is not sufficient to cause this disease.

Although IL-6 was first discovered for its key role in B cell differentiation to antibody-forming plasma cells, later studies showed that IL-6 is a pleiotropic cytokine which plays a role in the regulation of the immune response, the acute phase reaction, and haematopoiesis and is produced by a variety of cells. T cells, B cells, synoviocytes and chondrocytes have been identified as sources of IL-6 within the joint (Hirano et al 1988, Guerne et al 1989, Shinmei et al 1989). The abnormal production of IL-6, which has multiple biological effects which may be of relevance to the pathogenesis of RA, could explain several features observed in patients with RA, i.e. polyclonal plasmocytosis, presence of autoantibodies, increases in acute phase proteins and platelets and high levels of agalactosyl IgG.

6.1.2 TNF-α

TNF-α is produced mainly by cells of the monocyte/macrophage lineage, although vascular endothelial cells and lymphocytes in rheumatoid joints also produce TNF-α. TNF-α has been detected in serum samples in patients with RA, where levels correlate with disease activity (Vreugdenhil et al 1992).
TNF-α is also found at high concentrations in the synovial fluid in RA and there is evidence that it is locally produced (Husby and Williams 1988, Chu et al 1991).

In the murine collagen type II model of arthritis, the intra-articular administration of TNF-α leads to acceleration of disease (Cooper et al 1992) while treatment with anti-murine TNF-α antibodies significantly reduces inflammation and tissue destruction (Williams et al 1992). Clinical trials employing a monoclonal anti-TNFα antibody have demonstrated a dose-dependent efficacy in the treatment of human RA with impressive improvement in disease activity and acute phase responses lasting several weeks (Elliott et al 1993, Elliott et al 1994a,b).

Further support for a pathogenic role of TNF-α in arthritis has come from a study in transgenic mice (Keffer et al 1991). These mice expressed the human TNF-α gene modified by replacing the 3' untranslated sequence with that of the β globin gene, which had the effect of stabilizing mRNA expression and making it no longer macrophage-specific. Such mice developed a polyarthritis typified by cartilage destruction, bone erosion and leukocyte infiltration. Furthermore these pathogenic changes were totally inhibited by the administration of monoclonal anti-human TNF-α antibodies from birth. Interestingly, these transgenic mice developed no other pathology apart from arthritis. No studies have been reported on the level of IgG galactosylation in this line of TNF-α transgenic mice.

Preliminary studies in a different line of human TNF-α transgenic mice, in this case with expression targeted to T cells, showed an increase in the level of agalactosyl IgG with age. In this line, the 3' modified TNF-α gene/human β globin construct is under the control region of the human CD2 gene (Probert et al 1993). The TNF-α gene was expressed exclusively in the thymus, lymph nodes and spleen. Both the membrane-associated and secreted forms were detected. The normal pattern of T cell development in the thymus was
affected and the mice developed a lethal wasting syndrome, widespread vascular thromboses and necrosis of many organs.

TNF-α has also been proposed as one of the cytokines involved in regulation of glycosylation (Mackiewicz et al 1989b). In addition, several transcription factors are now known to be cytokine inducible, and in some cases, several different transcriptional factors can be induced by a single cytokine. The hypothesis that the cytokines IL-6 and TNF-α may induce alterations in IgG galactosylation via transcriptional regulation of the β1,4-GalTase gene was tested in mice transgenic for IL-6 and TNF-α. The direct effect of these cytokines on β1,4-GalTase mRNA levels in human PBL was also investigated in in vitro studies.

6.1.3 CD4+ T cells

There is considerable evidence supporting a role for CD4+ T cells in the pathogenesis of rheumatoid arthritis. RA synovial membrane is characterised by an infiltration of T cells, predominantly of the CD4+ helper/inducer phenotype (van Boxel and Paget 1975, Duke et al 1982, Burmester et al 1981). Increased numbers of IL-2R+ T cells are found in the peripheral blood of RA patients (Burmester et al 1981). In addition, the epidemiological association between RA and HLA DR4 and other DR haplotypes supports the hypothesis that the synovial CD4+ T cells are interacting with antigen-presenting cells in a specific immune response (Stastny 1978, Schiff et al 1982, Nepom et al 1989, Wordsworth et al 1989). Clinical improvement in patients with RA following the induction of peripheral blood lymphopaenia by thoracic duct drainage (Paulus et al 1977), total lymphoid irradiation (Strober et al 1985) and administration of cyclosporine (Tugwell et al 1990) provide further support for the importance of T cells in the development of RA. The use of anti-CD4 antibodies in the treatment of RA has been less impressive, resulting in only short-term clinical improvement and not long-term remission (Horneff et al 1991, Moreland et al 1995), although a recent study suggests
that the antibody dosage and treatment regimen are important in clinical benefit and the percentage of antibody-coated CD4\textsuperscript{+} lymphocytes in synovial fluid may be a predictor of clinical outcome (Choy et al 1996).

The importance of CD4\textsuperscript{+} T cells has been demonstrated in several murine models of the human disease. T cell clones bearing the CD4 membrane marker have been obtained from several animal models of arthritis and shown to transfer the disease to normal recipients (Holmdahl et al 1985, Stanescu et al 1987). It has also been possible, with the use of antibody directed against CD4, to delay or prevent arthritis in a number of animal models, including collagen-induced arthritis (Ranges et al 1985, Nagler-Anderson et al 1986), pristane-induced arthritis (Levitt et al 1992), streptococcal cell wall induced arthritis (Van den Brock et al 1992) and the spontaneous arthritis which develops in MRL lpr/lpr mice (Gilkeson et al 1992).

Not much is known, however, about the role of the T cell in the control of IgG glycosylation. Previous reports on mice lacking CD4\textsuperscript{+} T cells indicated the occurrence of immunoglobulin class switching from IgM to IgG (Rahemtulla et al 1994). Therefore, the existence of CD4 knockout mice provides an opportunity to investigate the role of this subset of T cells on IgG galactosylation \textit{in vivo}. The CD4 knockouts we have used in this study have been generated by introducing a disrupted CD4 gene into MRL lpr/lpr mice. The effect of CD4-deficiency on IgG galactosylation was, therefore, studied in a strain of mice which is already known to have abnormally galactosylated IgG (Figure 3.7).
6.2 RESULTS

6.2.1 The effect of IL-6 on \( \beta1,4\)-GalTase gene expression

6.2.1.1 IL-6 transgenic mice

Sera from 15 IL-6 transgenic mice and 17 control littermates were analysed for the concentration of IgG and the level of IgG galactosylation. Tissue from representative animals only was available to us for the analysis of mRNA (6 IL-6 transgenics and 4 control littermates) and enzyme activity (6 IL-6 transgenics and 6 control littermates) levels. All the mice used in this study were 3-months old.

a) IgG Concentration and galactosylation

The total IgG concentration was found to be 15-fold higher in the transgenics (n=12, 7 male and 5 female) compared to littermates (n=14, 8 male and 6 female). The level of IgG galactosylation, as measured by the ratio of BSI over RCAI binding, was found to be significantly lower in the transgenics compared to littermates (Figures 6.1 and 6.2).

b) \( \beta1,4\)-GalTase enzyme activity

The level of \( \beta1,4\)-GalTase enzyme activity was found to be similar in mice expressing the IL-6 transgene (n=6, 4 male and 2 female) and the controls (n=6, 4 male and 2 female). Serum was available only from three IL-6 transgenic mice and three control littermates for measuring the level of IgG concentration and galactosylation. These samples are shown with solid circles in Figure 6.3.
Figure 6.1 Serum IgG concentration in IL-6 transgenics and littermates. Mean ± standard error of the mean is indicated. Solid circles represent samples for which mRNA and enzyme activity data were obtained.

Figure 6.2 Level of agalactosyl IgG in sera from IL-6 transgenics and their littermates. Mean ± standard error of the mean is indicated. Solid circles represent samples for which mRNA and enzyme activity data were obtained.
c) $\beta 1,4$-GalTase gene expression

The mRNA levels for $\beta 1,4$-GalTase was also similar in IL-6 transgenics (n=6, 4 male and 2 female) and controls (n=4, 3 male and 1 female) using two different probes, GAPDH and $\beta$-actin, as internal controls to normalise for the amount of RNA in each lane (Figure 6.4a and 6.4b). Since fluctuations in GAPDH mRNA levels were suspected in initial experiments, $\beta$-actin was also included in the RPAs as a second internal control.
Figure 6.4a  RNase protection analysis of splenic β1,4-GalTase RNA in IL-6 transgenic mice (lanes 1, 4-6) and their littermates (lanes 2, 3 and 7).

Controls: yeast tRNA hybridised with β-actin probe alone (A), β1,4-GalTase probe alone (B) and GAPDH probe alone (C) without RNase, showing full length 350-nt β-actin probe, 602-nt β1,4-GalTase probe and 404-nt GAPDH probe respectively. Lanes D, E and F show yeast tRNA hybridised separately with β-actin (D), GAPDH (E) and β1,4-GalTase (F) and treated with RNase to show the protected fragments for β-actin (250 nt), GAPDH (316 nt) and β1,4-GalTase (559 nt) respectively.
Figure 6.4b  Quantitation of β1,4-GalTase mRNA levels in spleen cells by phospho-imaging (6 transgenics and 4 control littermates). Results are expressed as the ratio of β1,4-GalTase over β-actin (a) and β1,4-GalTase over GAPDH (b) mRNA signal. Mean ± standard error of the mean is indicated. Solid circles represent samples for which enzyme activity, IgG concentration, and IgG galactosylation data were obtained.

6.2.1.2 In vitro studies
The effect of IL-6 on β1,4-GalTase gene expression in cultured human peripheral blood lymphocytes (i.e. PBMCs depleted of plastic-adherent cells) was determined at specific time points. No changes in β1,4-GalTase mRNA levels were detected with the addition of IL-6 (250 units/ml) to the cell cultures (Figure 6.5a and 6.5b). Due to the limited number of cells available, two separate experiments were carried out using cells from two individuals.
Figure 6.5  β1,4-GalTase mRNA levels in human peripheral blood lymphocytes cultured in the presence or absence of recombinant human IL-6 for 0.5-3.0 hours (Figure 6.5a) and 5.0-120 hours (Figure 6.5b). Error bars indicate ± standard error of the mean for duplicate RPA measurements on each sample.
6.2.2 The effect of TNF-α on β1,4-GalTase gene expression

6.2.2.1 TNF-α Transgenic mice

Eight TNF-α transgenic mice (3 male, 5 female) and 9 control littermates (2 male, 7 female) were used in this study. Previous unpublished studies by K. Bodman indicated raised agalactosyl IgG levels in 18-week old mice (4 transgenics and 3 controls) but not in 10-week old mice (6 transgenics and 9 controls) only in older mice. Therefore, the mice used in this study were analysed at 19±1 weeks of age.

a) IgG Galactosylation

The level of IgG galactosylation was found to be similar in CD2/TNF transgenics (n=8) and their littermates (n=9) (Figure 6.6).

![Figure 6.6](image-url)

Figure 6.6 Level of agalactosyl IgG in sera from CD2/TNF transgenics and their littermates (19 ±1 weeks old). Mean ± standard error of the mean is indicated.
b) \( \beta 1,4GalTase \) gene expression

The expression of the \( \beta 1,4GalTase \) gene was comparable in RNA from spleen tissue of transgenics (n=8) and of their littermates (n=9) (Figure 6.7).

![Graph showing mRNA levels of \( \beta 1,4GalTase \) in transgenics and littermates](image)

**Figure 6.7** \( \beta 1,4GalTase \) mRNA levels in spleen tissue using RPA (8 transgenics and 9 control littermates). Results are expressed as the ratio of \( \beta 1,4GalTase \) over GAPDH mRNA signal. Mean ± standard error of the mean is indicated.

6.2.2.2 In vitro studies

The effect of recombinant human TNF-\( \alpha \) on \( \beta 1,4GalTase \) mRNA levels was determined using normal human PBL cell cultures. Comparable mRNA levels were found in cultures with or without additional TNF-\( \alpha \) (20 ng/ml). Figure 6.8 shows the result of two separate experiments (a and b) carried out using PBL from two individuals.
Figure 6.8  β1,4-GalTase mRNA levels in human peripheral blood lymphocytes cultured in the presence or absence of recombinant human TNF-α for 0.5-3.0 hours (Figure 6.8a) and 5.0-120 hours (Figure 6.8b). Error bars indicate ± standard error of the mean for duplicate RPA measurements on each sample.
6.2.3 T-cell control of IgG glycosylation

6.2.3.1 CD4-Knockouts

Homozygous CD4-deficient MRL *lpr/lpr* mice were compared to heterozygous CD4-expressing littermates in two age groups with regard to the concentration and galactosylation of serum IgG. In the 4-months old group, 6 CD4-expressing and 6 CD4-deficient mice, and in the 8-months old group 4 CD4-expressing and 6 CD4-deficient mice were analysed.

a) IgG Concentration

The total IgG concentration was determined in MRL *lpr/lpr* CD4 KO mice and the controls. In 4-months old mice, the IgG concentration was significantly lower in mice lacking CD4 (*p*<0.0001). This difference was not seen in 8-months old mice (*p* > 0.24, Figure 6.9).

![Figure 6.9](image_url)  
Figure 6.9 Serum IgG concentration in 4-months old CD4-expressing (n=6) and CD4-deficient (n=6) mice and also in 8-months old CD4-expressing (n=4) and CD4-deficient (n=6) mice. Mean ± standard error of the mean is indicated.
b) IgG Galactosylation

No difference in the level of IgG galactosylation was observed between CD4-expressing and CD4-deficient mice (p>0.31 and 0.30 for 4-months old and 8-months old mice respectively). Furthermore, the level of IgG galactosylation of 8-months old mice was comparable to that of 4-months old mice (p>0.09 and p> 0.16 for CD4-expressing and CD4-deficient mice respectively, Figure 6.10).

![Graph showing serum agalactosyl IgG levels in 4-months old CD4-expressing (n=6) and CD4-deficient (n=6) mice and also in 8-months old CD4-expressing (n=4) and CD4-deficient (n=6) mice. Mean ± standard error of the mean is indicated.]

Figure 6.10  Serum agalactosyl IgG levels in 4-months old CD4-expressing (n=6) and CD4-deficient (n=6) mice and also in 8-months old CD4-expressing (n=4) and CD4-deficient (n=6) mice. Mean ± standard error of the mean is indicated.
6.3 DISCUSSION

6.3.1 IL-6

The significant increase in agalactosyl IgG levels in IL-6 transgenic mice confirms the previously published results by Rook and colleagues (1991a) and could reflect an effect of IL-6 on the IgG glycosylation in B-cells. The influence of IL-6 on B-cells could be direct or indirect through the release of other cytokines, hormones and regulatory agents. Although IL-6 was first identified for its action on B-cell differentiation and immunoglobulin induction (Hirano et al 1986), the precise mechanism by which IL-6 acts has not yet been established.

A significant increase in serum IgG concentration was detected in these IL-6 transgenic mice. This was reported previously by Suematsu and colleagues and IgG1 was found to be the major polyclonally increased immunoglobulin heavy chain class, being increased 120- to 400-fold as compared to those of age-matched control normal mice (Suematsu et al 1989). The other classes of immunoglobulins (IgM, IgG2a, IgG2b, IgG3 and IgA) were found to remain within three times of the immunoglobulin concentrations in normal mice. A recent study (Williams and Rademacher 1996) has reported murine IgG isotype specific differences in galactosylation with IgG1 exhibiting the highest %G0 (45-48%) followed by IgG2a (27-37%), IgG3 (20-32%) and IgG2b the lowest (13-17%). Therefore, it is possible that the increase in agalactosyl IgG observed in IL-6 transgenic mice, as measured by the BSII : RCAl binding to IgG captured on protein G' in this study and by anti-GlcNAc reactivity of protein A-captured IgG previously by Rook et al, is entirely due to increased IgG1 isotype production, and not due to any change in the galactosylation of IgG per se.

No cytokine response elements have been detected in the promoter region of the β1,4-GalTase gene. However, three glucocorticoid response elements and binding sites for other regulatory nuclear binding factors are present in
the 5'-flanking region of the β1,4-GalTase gene. (Harduin-Lepers et al. 1993). We found no significant difference in the expression of the β1,4-GalTase gene between IL-6 transgenics and the controls. Enzyme activity of β1,4-GalTase was also found to be similar in IL-6 transgenics and controls. Unfortunately, matching sera were not available to directly correlate levels of agalactosyl IgG with β1,4-GalTase mRNA and enzyme activity for all the samples. However, there were 6 mice (3 transgenics and 3 controls) for which IgG galactosylation, IgG concentration, β1,4-GalTase mRNA and enzyme activity data were obtained (as shown by solid circles in figures 6.1, 6.2, 6.3, and 6.4b). As seen in figures 6.1 and 6.2, the difference (between the transgenics and controls) in the level of IgG galactosylation (p>0.41) and IgG concentration (p>0.27) in this group of six mice do not represent the difference observed for the whole population (p>0.005 for IgG galactosylation and p<0.0001 for IgG concentration). Therefore, β1,4-GalTase mRNA and enzyme activity levels have to be measured in more IL-6 transgenic and control mice before a conclusion can be reached. The results of this study (taken as it is) would suggest that the undergalactosylation of IgG in IL-6 transgenics is caused by mechanisms other than down-regulation of β1,4-GalTase.

Studies performed to determine whether IL-6 can directly modulate the β1,4-GalTase transcriptional level in vitro have shown negative results. One explanation for this could be that for cells to respond to IL-6, they need to be activated. There is evidence that IL-6R is expressed on activated B cells, but not on resting B cells (Hirata et al. 1989, Taga et al. 1987). Length of incubation time and concentration of IL-6 are other factors needed to be considered. The addition of IL-6 (100 units/ml) to a human myeloma cell line (OPM-1) has been shown to result in a slight decrease in β1,4-GalTase activity (Nakao et al. 1990). The effect of IL-6 (100 units/ml) on IgG galactosylation has also been explored by K. Bodman through in vitro studies. IL-6 (100 units/ml) added to LPS-stimulated splenic mononuclear cell cultures from non-arthritic (n=7) and collagen induced arthritic male DBA/1 mice (n=8)
did not show any significant change in de novo secreted agalactosyl IgG levels in cultures from either group (Bodman 1995). However, IL-6 (100 units/ml) added to an IgG-secreting EBV-transformed cell line (HB-1), resulted in an increase in the level of agalactosyl IgG (Bodman 1995).

6.3.2 TNF-α

Preliminary experiments performed by K. Bodman showed an increase in agalactosyl IgG levels in sera from CD2/TNF transgenics compared to littermates at 18 weeks of age but comparable levels at 10 weeks of age. In the present study, however, no differences in the level of agalactosyl IgG nor in the mRNA levels for the β1,4-GalTase gene in CD2/TNF transgenes were seen compared to littermate controls. The transgenic mice taken for this study (19±1 weeks old) showed the wasting syndrome, and the attempt to make splenic lymphocyte suspension was unsuccessful, giving a low yield and poor viability. Histological studies have shown previously that lymphoid organs in these transgenic mice show an overall depletion of lymphocytes and an absence of lymphatic/splenic nodules (Probert et al 1993).

Probert and colleagues showed that in this line of transgenic mice high levels of human TNF-α mRNA were found exclusively in the thymus, lymph nodes and spleen, while high levels of TNF-α protein were detectable in the serum (Probert et al 1993). Why these mice and the mice expressing human TNF-α transgenes driven by wild type promoters did not show any sign of arthritis, while the TNF-globin transgenic mice did (Keffer et al 19991) remains unclear. It is possible that differences in cell types expressing the transgene may be responsible for the joint pathology.

The in vitro studies performed here showed no effect of TNF-α on β1,4-GalTase gene expression within the time period studied. This supports the results obtained for the TNF-α transgenic mice. As in the in vitro IL-6 studies, no control test has been run to check the bioactivity of the cytokines used in
the experiments, although both IL-6 and TNF-\(\alpha\) had just been obtained from Genzyme and IL-6 was also known to be functional in other experiments.

The effect of anti-TNF-\(\alpha\) antibody treatment in RA patients on the level of IgG galactosylation has been investigated by Rankin et al (1995). Comparable levels of serum agalactosyl IgG were observed in RA patients receiving anti-TNF-\(\alpha\) antibody (n=24) and placebo (n=12) before and 8 weeks after starting the treatment. No significant change in agalactosyl IgG levels was seen even in those patients who showed significant improvement in parameters of disease activity (n=8). Although small patient numbers and the the reduced effect of the drug by week 8 have been suggested by the authors as the possible reasons for the lack of a significant change in agalactosyl IgG levels in this study, the data obtained so far points to a lack of association between the levels of TNF-\(\alpha\) and serum IgG galactosylation.

### 6.3.3 A role for CD4* T cells in IgG galactosylation

The production of IgG was significantly decreased at 4 months of age in the CD4-deficient mice compared to heterozygous controls. It is less easy to draw solid conclusions for the 8-month old mice due to the very small number of samples tested for the CD4-expressing group. Surprisingly, however, even in the absence of CD4, MRL Ipr/lpr mice are able to make moderate levels of IgG. It has been shown previously by others that CD4 is not absolutely necessary for effector function of class II MHC-restricted helper T cells (Rahemtulla et al 1994, Locksley et al 1993). Rahemtulla and colleagues have demonstrated that C57BL/6 X 129/sv (H-2^b) mice lacking CD4 show in vivo immunoglobulin isotype class switching from IgM to IgG in response to sheep erythrocyte and vesicular stomatitis virus (VSV). A population of CD4^- TcR alpha beta^ T cells was shown to be responsible for providing help in the antibody response of CD4-deficient mice to VSV infection (Rahemtulla et al 1994).
Deficiency of CD4 in MRL \textit{lpr/lpr} did not influence the level of agalactosyl IgG, as measured by the binding of BSII/RCAI in either age group, suggesting that IgG galactosylation was not controlled by CD4 cells in these mice. On the other hand, the level of terminal galactose (BSII/RCAI binding) on IgG did not decrease with age in MRL \textit{lpr/lpr} mice in this experiment, regardless of expression or deficiency of CD4. A significant increase in agalactosyl IgG with age has previously been observed in almost all strains tested including MRL \textit{lpr/lpr} mice (Bodman \textit{et al} 1994). Differences observed could be due to the small number of animals in the two studies. In addition, the control mice used in the present study are heterozygous littermates and wild-type MRL \textit{lpr/lpr} mice (i.e. homozygous for CD4-expression) are not included in the study. Despite showing a massive lymphoproliferation comparable to that in control mice at 5-months of age, the CD4-deficient MRL \textit{lpr/lpr} mice were found not to develop autoimmunity, as evident by the significant decrease in the mean titre of antibodies to ds DNA in these mice ($p<0.001$, Chesnutt \textit{et al} 1995). Although the CD4-deficient and -expressing mice in our study showed comparable levels of agalactosyl IgG, the concentration of total serum IgG was significantly lower in the CD4-deficient mice. To what extent the anti-ds DNA antibodies and agalactosyl IgG overlap remains to be shown.

A significant decrease in the level of agalactosyl IgG has been observed in NZB mice ($n=3$) treated with a non-depleting anti-CD4 monoclonal antibody at five weeks post-treatment compared with PBS-injected mice ($n=3$) (Bodman 1995). In another preliminary experiment, Bodman showed that addition of T-cell enriched RA PBMC to B-cell enriched normal control cells resulted in the production of supernatants, with relatively increased levels of IgG G0 when compared to autologous mixes. The reverse was seen on addition of control T-cell enriched PBMC to B-cell enriched RA cells. Although these experiments were all of a preliminary nature and carried out in completely different systems, taken together, the changes observed suggest a contribution of T-cells to the regulation of IgG galactosylation. The CD4$^+$ T
cells may be regulating IgG glycosylation either by producing cytokines themselves, or by stimulating other cells (through interaction with professional and non-professional antigen presenting cells) to secrete cytokines. Since most of the cytokines in the RA joint, such as IL-6 and TNF-α, are of synoviocyte or macrophage origin (Guerne et al 1989, Field et al 1991) and not from T cells, the latter hypothesis seems more plausible.
CHAPTER 7

THE EFFECT OF CELLULAR ACTIVATION ON β1,4-GALTASE GENE EXPRESSION
7.1 INTRODUCTION

The role of cellular activation in the regulation of β1,4-GalTase mRNA was investigated using *in vitro* culture of human peripheral blood lymphocytes. The stimulatory agents used in these experiments included phytohaemagglutinin (PHA, a T-cell mitogen), pokeweed mitogen (PWM, mitogenic for both T and B cells), phorbol 12-myristate 13-acetate (PMA, a phorbol ester), ionomycin (a calcium ionophore), and forskolin (a cAMP-inducer).

PHA is an extract from the red kidney bean (*Phaseolus vulgaris*) and PWM has been isolated from the roots of *Phytolacca americana*. The lectins PHA and PWM bind to carbohydrate residues (*N*-acetylgalactosamine and di-*N*-acetylchitobiose, respectively) in glycoproteins and are able to cross-link glycoproteins on the surface of cells, although the exact mechanism of action of these lectins is still not clear. PHA is one of the most potent T cell mitogens and it has been suggested that PHA stimulates T cell activation by engaging and cross-linking the T-cell antigen receptor complex together with other T cell surface molecules (Geppert 1992). PWM has been shown to stimulate cell growth of both T and B cells and induce Ig production in peripheral blood lymphocytes. PWM-induced Ig production is found to be dependent on both T cells and macrophages (Horii and Hirano 1992).

Phorbol esters are also plant products which bind to protein kinase-C and activate it directly (Evans and Farrar 1992). Calcium ionophores such as ionomycin are pharmacological agents which when added to intact cells mimic the Ca^{2+}-mediated signal of the inositol phospholipid signalling pathway. Forskolin induces cAMP levels which affect cells by stimulating cAMP-dependent protein kinase (A-Kinase) ultimately leading to cellular responses through a cascade of phosphorylation events. In some cells an increase in cAMP activates the transcription of specific genes, such as somatostatin. The promoter region of these genes contain a short DNA
sequence which is recognised by a specific gene regulatory protein that activates transcription from these genes when it is phosphorylated by A-kinase (Alberts et al 1989).

The aim of the experiments reported in this chapter was to identify factors which positively and negatively regulate β1,4-GalTase expression and to attempt to correct, in vitro, the IgG glycosylation defect in RA B-cells. It should be pointed out that, due to time constraints, the data presented are of a preliminary nature.
7.2 RESULTS

Peripheral blood lymphocytes (monocyte-adhered PBMCs) cultured with PHA (1 μg/ml), PWM (1 μg/ml), or PMA (0.1 μg/ml) separately for 6, 18, and 72 hours showed little change, if any, in the level of β1,4-GalTase mRNA. However, the level of GAPDH mRNA, which had been used with the initial intention of acting as an internal control to normalise for the amount of RNA in each lane, changed drastically following cellular activation by all three mitogens (Figure 7.1). This interpretation of the results is valid only if the amount of RNA is equivalent in each lane, as initially estimated by spectrophotometric methods.

At the time of obtaining these results, a study published by Bhatia et al (1994) investigated the expression of GAPDH and 28S ribosomal RNA genes in a variety of mouse tumourigenic and metastatic cell lines. They showed that while GAPDH mRNA levels varied markedly amongst these cell lines and were elevated compared to the normal parental mouse cell line, the levels of 28S ribosomal RNA did not significantly vary amongst the malignant cell lines and were approximately at the same level as that found in the normal parental mouse cell line. Therefore, an 18S rRNA probe, which had recently been commercialised, was provided and used as an internal standard for the amount of RNA loaded in each lane. Figure 7.2 shows that the mRNA levels for β1,4-GalTase are reduced upon culture with no added stimuli. Stimulation with either PMA or PHA did not rescue this reduction in the level of β1,4-GalTase mRNA either after 18- or 72-hour of cell incubation. In this particular experiment there was an indication that cells stimulated with PWM showed no decrease in the level of β1,4-GalTase mRNA after 18 hours in culture (data was not obtained after 72 hour of culture in this experiment). Up-regulation of GAPDH mRNA with PWM, PHA, and PMA showed that the reagents used in this study were active.
**Figure 7.1** The levels of β1,4-GalTase mRNA as measured by the RPA in normal peripheral blood lymphocytes stimulated with PWM, PHA, or PMA for 6 (lanes 1-4), 18 (lanes 5-8), or 72 hours (lanes 9-12). The control population (Nil) consisted of the same cells incubated in medium only for the specified period of time. 4 and 8 μg of total RNA were used in this study. The result for 4 μg only is shown in this figure.

**Controls:** Lane A, human lymphocyte RNA hybridised with β1,4-GalTase probe with addition of RNase, showing 150-nt protected fragment. Lane B, human lymphocyte RNA hybridised with GAPDH probe with addition of RNase, showing 316-nt protected fragment. Lane C, human lymphocyte RNA hybridised with p58°^TA probe with addition of RNase, showing the protected fragment (p58°^TA expression was not followed in this study). Lanes D-F, increasing amounts of total RNA from human lymphocytes (4, 8, and 16 μg, respectively) hybridised with the same amount of probes, showing that the probes are in molar excess. Lane G, yeast tRNA hybridised with all three probes with addition of RNase. Lanes H-J, yeast tRNA hybridised with β1,4-GalTase, GAPDH, and p58°^TA probes respectively, without RNase, showing full length 347-nt β1,4-GalTase and 404-nt GAPDH probes.
Figure 7.2 Quantitation of the β1,4-GalTase mRNA levels using RPA for human peripheral blood lymphocytes stimulated with PHA, PWM, and PMA for 18 and 72 hours. The control samples represent the same cells with no added stimuli.

However, when the experiment was repeated with human PBLs in the presence and absence of PWM for 0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 18, and 72 hours, comparable levels for β1,4-GalTase mRNA were observed (Figure 7.3).

It would thus appear that the level of β1,4-GalTase mRNA declines during lymphocyte cell culture, and that this decline occurs irrespective of stimulation by mitogens or direct activation of protein kinase-C.
Figure 7.3  Quantitation of the β1,4-GalTase mRNA levels for human peripheral blood lymphocytes stimulated with PWM for specified time points. Error bars indicate ± standard error of the mean for two RPA measurements.

The effect of the calcium ionophore, ionomycin, on β1,4-GalTase mRNA levels in human PBLs cultured for 0-5 hours was also studied. The time period of 0-5 hours was chosen because the β1,4-GalTase gene has been classified as an "early" gene (discussed below). The results of two separate experiments are shown in figure 7.4a and 7.4b. An apparent increase in β1,4-GalTase mRNA levels was seen after 0.5 hours in one of the two experiments.
Figure 7.4  
β1,4-GalTase mRNA levels for human PBL stimulated with calcium ionophore ionomycin for specified time points (experiment a, 0-5 hours; experiment b, 0-3 hours). Error bars indicate ± standard error of the mean for two RPA measurements.
The levels of PBL β1,4-GalTase mRNA were also measured following the addition of forskolin, a cAMP inducer. Cells were harvested at 0, 0.5, 1.0, 2.0, 3.0, and 4.0 hours (Figure 7.5). Although the β1,4-GalTase mRNA level for cells stimulated with forskolin for 2 hours appears higher than that of the relative control, fluctuations in the level of β1,4-GalTase mRNA in the control cells suggest no significant change in β1,4-GalTase mRNA levels upon stimulation with forskolin in this single experiment.

Figure 7.5  β1,4-GalTase mRNA levels for human PBL stimulated with forskolin, a cAMP inducer, for specified time points. Quantitation by densitometry of the results of one RPA is shown.
7.3 DISCUSSION

The 5'-flanking region of the β1,4-GalTase gene has an extremely GC-rich sequence, as is typical of housekeeping-type genes, as well as oncoproteins, growth factors, and receptor proteins (Kozak 1992). Although the sequence of the 5' flanking region of the β1,4-GalTase gene has been found to contain potential transcriptional regulatory elements, including ten SP1 binding sites, six AP2 binding sites, three GRE (glucocorticoid response element), and a CRE (cyclic AMP response element), detailed analysis showed that the putative AP2 and CREB (CRE-binding protein) sites within this region are non-functional (Harduin-Lepers et al. 1993). Investigations by this group failed to find any promoter activity within the 5'-region flanking the β1,4-GalTase gene. However, a weak promoter activity was expressed when the SV40 enhancer element, which has been shown to be useful for detecting weak endogenous promoters, was used (subcloned into the β1,4-GalTase construct).

In in vitro stimulation experiments, an important consideration is the time necessary for the gene of interest to respond to the stimulating agent. The β1,4-GalTase gene has been found to show a temporal expression during the cell cycle (Masibay et al. 1991, Pouncey et al. 1991). The activation of cell-cycle dependent genes (Denhardt et al. 1986) represents a primary response of the cell to serum or growth factors. Depending on their time of maximal expression during the transit from G₀ to S, these genes have been categorised as immediate early, early, and late genes. The "immediate early" genes are expressed within one hour of a transit of the cell from G₀ to S and they generally code for nuclear transcriptional factors. The genes whose maximum expression occurs within 2-4 hour of serum stimulation have been categorised as “early” genes. Immediate early and early genes do not require de novo protein biosynthesis and require only the activation of pre-existing transcriptional factors for the expression of their mRNA during cellular proliferation. Based on these criteria, the β1,4-GalTase gene was classified
as an "early" gene in a study by Masibay and colleagues (1991), in which an increase of 3-4-fold in the level of β1,4-GalTase transcript was found 2-4 hours after serum-stimulation of quiescent 3T3 cells followed by a decline 8-12 hours post-stimulation (Masibay et al 1991). In addition, they found that β1,4-GalTase mRNA induction by serum was enhanced in the presence of the protein synthesis inhibitors cycloheximide or anisomycin.

Pouncey et al (1991) also obtained evidence that β1,4-GalTase gene expression is regulated during the normal cell cycle, being low in the quiescent cells (G₀) and high during late G₁, S, and early G₂ phase of the cell cycle. The authors of this paper also suggested that this manner of expression of the β1,4-GalTase gene upon re-entry into the cell cycle from quiescence resembles that of "early response" genes (Pouncey et al 1991). However, the β1,4-GalTase gene was classified as a "late" gene by Kudo and Narimatsu in a study in which the increase in β1,4-GalTase mRNA during differentiation of F9 cells was observed after 2 days and the maximum increase was observed after 8 days of exposure of the cells to retinoic acid and dibutyryl cAMP (Kudo and Narimatsu 1995). Differences in the cell type and/or the activating agent used might have been involved in the differential regulation of the β1,4-GalTase gene in these studies. Based on the available information and the limited number of cells available, we chose short incubation times for investigating the effect of stimulating agents on β1,4-GalTase gene expression. Since mitogens are known to require longer time periods to affect cellular proliferation, the longer incubation times of one- and three-days were also included in the experiments using PHA, PWM, and PMA.

Within the conditions of the experiments conducted, we did not find any noticable effect on β1,4-GalTase gene expression with the activating agents used. Other reports on the effect of several growth factors on β1,4-GalTase mRNA expression in 3T3 cells have been similar (Masibay et al 1991). Insulin (1μg/ml), epidermal growth factor (EGF, 10 ng/ml), fibroblast growth factor (50 ng/ml), vasopressin (10 ng/ml), 12-O-tetradecanoylphorbol-13-acetate
(TPA, 50 ng/ml), calcium ionophore A23187 (5 μM), and dibutyryl cAMP (2μM) did not show any effect on β1,4-GalTase gene expression after 2-hour stimulation in culture. Cholera toxin (100 ng/ml), however, which causes a prolonged elevation in intracellular cAMP levels (Alberts et al. 1989), was the only agent that was reported to enhance the level of β1,4-GalTase mRNA in 3T3 cells (Masibay et al. 1991).

Roth et al. (1991) reported that forskolin- or 2-chloroadenosin-induced stimulation of PC12 cells leads to elevated β1,4-GalTase enzyme activity. The effect was seen as early as 3 hours after stimulation and maximal activity was reached at approximately 12 hours. It was speculated in their paper that the β1,4-GalTase gene might be regulated by CREB via cAMP-dependent protein kinase (A-kinase), since PC12 cells have high levels of endogenous CREB.

Treatment of mouse F9 tetracarcinoma cells with retinoic acid and retinoic acid plus dibutyryl cAMP for 8 days has been demonstrated to increase the amount of β1,4-GalTase mRNA ~1.5 and 6.5 fold respectively (Kudo and Narimatsu 1995). It was demonstrated that the increase in mRNA was not due to a rise in the transcriptional activity of the β1,4-GalTase gene and that the rate of de novo synthesis of the β1,4-GalTase gene transcript in the retinoic acid/dibutyryl cAMP-induced cells was almost the same as in undifferentiated F9 cells. No cis-elements responding to either retinoic acid-mediated or dibutyryl cAMP-mediated activators were found in the upstream region of the β1,4-GalTase gene in this study. Measurement of the stability of β1,4-GalTase transcripts, however, revealed that their half-life gradually increased as the duration of retinoic acid/dibutyryl cAMP exposure increased. Therefore, post-transcriptional stabilisation, and not transcriptional activation was found to be the mechanism of β1,4-GalTase gene regulation during differentiation of mouse F9 tetracarcinoma cells. cAMP-regulated mRNA stabilisation has also been reported for several other mRNA species, such as lactate dehydrogenase and renin, however little is known about the
mechanism of cAMP-mediated mRNA stabilisation. The experiments carried out by Roth et al (1991), Kudo and Narimatsu (1995), and Masibay et al (1991) suggests that prolonged exposure to cAMP may be needed to affect the β1,4-GalTase mRNA levels. This may be the reason that forskolin did not show much effect on β1,4-GalTase mRNA levels within 4-hours incubation with peripheral blood lymphocytes in the present study, and that dibutyryl cAMP did not change the β1,4-GalTase gene expression after 2 hours of stimulation in the study by Masibay et al (1991).
CHAPTER 8

GENERAL DISCUSSION
The reduced levels of IgG galacosylation in patients with RA has been suggested by a number of investigators to be associated with aberrant control of β1,4-GalTase enzyme activity in B-lymphocytes. Therefore, it was important to establish whether the structure and/or the expression of the β1,4-GalTase gene is altered in RA lymphocytes. The following were analysed:

1. The structure of the β1,4-GalTase genes in patients with RA and also in an animal model of the human disease, the MRL lpr/lpr. No gross structural alterations of the β1,4-GalTase gene were found in patients with RA, and the structure of the gene was shown to remain intact in the MRL lpr/lpr mice.

2. The levels of β1,4-GalTase gene expression in B-lymphocytes from patients with RA and normal individuals. These were shown to be comparable.

3. The levels of β1,4-GalTase mRNA in B cells from MRL lpr/lpr mice in comparison with the congenic strain MRL +/+ mice and also CBA/Ca strain mice (which possess significantly higher galactosylated IgG). Similar levels of β1,4-GalTase mRNA were demonstrated in B cells from all three strains.

4. β1,4-GalTase mRNA and enzyme activity levels in relation to the agalactosyl IgG levels in normal mice during pregnancy, a natural physiological state associated with a rise in IgG galactosylation. In spite of a significant rise in the galactosylation of IgG after mid-pregnancy and post-partum, no difference in the level of mRNA or the activity of the β1,4-GalTase enzyme was observed.

These observations clearly show that significant changes in IgG galactose levels can occur in the absence of concomitant changes to the steady state levels of lymphocytic β1,4-GalTase mRNA.
The correlation between β1,4-GalTase gene expression and agalactosyl IgG levels was further investigated in different B-lymphoblastoid cell lines showing variation in β1,4-GalTase enzyme activity, but all secreting highly galactosylated IgG. It was shown that these cell lines contained different amounts of β1,4-GalTase mRNA in spite of comparable levels of IgG galactosylation. The lack of correlation between β1,4-GalTase mRNA levels and the extent of galactosylation has also been shown in a study by Youakim and Shur (1993). It was revealed in that study that overexpression of β1,4-GalTase (3-fold) in F9 embryonal carcinoma cells had no significant effect on qualitative or quantitative biosynthesis of glycoproteins.

The information available regarding β1,4-GalTase and the reactions catalysed by this enzyme show the complexity of the galactosylation reaction:

1. The expression of β1,4-GalTase, as a trans-Golgi resident glycoprotein functioning in the biosynthesis of N-linked glycans, is controlled by a GC-rich promoter with multiple SP1 binding sites (Harduin-Lepers et al. 1993), suggesting that β1,4-GalTase is a housekeeping enzyme which is constitutively expressed. In fact, β1,4-GalTase-deficient cell lines derived from vertebrate animals have not been reported (Berger and Thurnher 1993).

2. The extent of potential secondary structure within the 5'-untranslated region of the β1,4-GalTase transcript predicts that β1,4-GalTase enzyme levels could additionally be regulated at the translational level (Harduin-Lepers et al. 1993).

3. Phosphorylation of β1,4-GalTase enzyme by the putative regulatory p58GTA protein kinase gene, as discussed earlier, has been shown to increase the activity of the enzyme (Bunnell et al. 1990).

4. The specificity of the β1,4-GalTase enzyme may be modified by α-lactalbumin to catalyse the synthesis of lactose.
5. Cationic activation of β1,4-GalTase by polyamines and by basic peptides and proteins in a manner analogous to the allosteric activation of the enzyme by α-lactalbumin has also been suggested (Navaratnam et al 1988).

6. The glycosylation reaction may be regulated by variations in the luminal concentration of Mn$^{2+}$ (Navaratnam et al 1992).

7. The pH of the Golgi may be involved in altering the glycosylation reaction catalysed by β1,4-GalTase for example by affecting Mn$^{2+}$ binding. Such a pH-sensitivity has been suggested for Con A (Sherry et al 1975) and hepatic arginase (Kuhn et al 1991).

8. Multimerization of β1,4-GalTase may enhance the activity of the enzyme. This has been suggested as a possible reason for increased protein levels (6-fold) on the sperm surface of β1,4-GalTase transgenic mice resulting in a synergistic effect on enzyme activity (20-30 fold) (Youakim et al 1994).

9. An imbalance in nucleotide pools may play a role in the regulation of glycosylation by modulating the β1,4-GalTase. Studies by Sharma et al (1993) showed that the mRNA and activity of β1,4-GalTase increase during liver cell proliferation following partial hepatectomy, or after the administration of lead nitrate, a liver mitogen. The addition of exogenous orotic acid (1%), a normal cellular constituent generated during the biosynthesis of pyrimidine nucleotides, to the diet of rats 2 weeks prior to partial hepatectomy inhibited the rise in β1,4-GalTase mRNA and enzyme activity 24 hours following surgery. The supplementation of 1% orotic acid diet with 0.3% adenine relieved the inhibition of β1,4-GalTase activity. Orotic acid is known to cause an imbalance in the pool sizes of nucleotides characterised by an increase in the level of uridine nucleotides and a decrease in the level of adenosine nucleotides. Adenine, which inhibits the metabolism of orotic acid to uridine nucleotides, counteracts the inhibitory
effect of orotic acid, supporting the idea that somehow the imbalance in the pool sizes of nucleotides affected the enzyme activity.

10. β1,4-GalTase activity has been shown to be modulated by lipids (Mitranic et al. 1983). While some lipids show a stimulatory effect (i.e. phosphatidylcholine), some inhibit the activity almost totally (phosphatidic acid and phosphatidylserine). In lipid mixtures, the effect of the stimulatory lipid predominates (Mitranic et al. 1983).

The factors listed above outline the possible mechanisms for the regulation of β1,4-GalTase enzyme activity. Any alterations in the association of β1,4-GalTase with its multiple ligands could affect the activity of the enzyme. These have to be taken into account when analysing the activity of β1,4-GalTase enzyme. For example, the assays employed to determine the enzyme activity levels reported in the literature have produced different results based on the acceptor substrate used and/or the method of cell preparation. Thus, comparable enzyme activity levels were observed in B-cells from RA patients and normal individuals when GlcNAc-pITC-BSA (Keusch et al. 1995), asialo-agalacto-transferrin, and asialo-ovine submaxillary mucin (Furukawa et al. 1990) were used as the acceptor substrate in the reaction mixture. This is suggestive of a difference at the level of enzyme activity regulation rather than defects in the β1,4-GalTase enzyme itself. The method of cell preparation has also been shown to affect the difference in enzyme activity levels between RA and control groups.

Kinetic studies carried out by Furukawa and co-workers (1990) indicate that β1,4-GalTase in liver homogenate may be more efficient at transferrin galactosylation than the enzyme in B-cell homogenate, and vice versa for IgG. The explanation for this was suggested by Furukawa and colleagues to be the presence of a B-cell specific β1,4-GalTase enzyme, however, only one gene has been identified for β1,4-GalTase so far. Alternatively, one could speculate that the cell-specific internal environment, such as intracellular
concentrations of basic peptides and proteins, has adapted the enzyme for a more efficient galactosylation of the major glycoprotein synthesised by that particular cell. Modification of β1,4-GalTase by α-lactalbumin in mammary tissue to use glucose more efficiently may be considered as such an example.

*In vitro* experiments examining the *de novo* secreted IgG from different lymphoid compartments of MRL *lpr/lpr* showed different levels of agalactosyl IgG being secreted from cells taken from different compartments. This is also suggestive of a role for the micro-environment in regulating the galactosylation of IgG. In addition, different culture methods have been shown to affect the glycosylation of human (Kumpel et al 1994) and murine (Patel et al 1992, Lund et al 1993) IgG monoclonal antibodies.

The possible involvement of the glycosylation regulating cytokines such as IL-6 and TNF-α was investigated in this thesis by examining the levels of agalactosyl IgG and β1,4-GalTase mRNA. TNF-α did not have any effect on IgG galactosylation or β1,4-GalTase mRNA levels in TNF-α transgenic mice and in human PBL cultures *in vitro*. Although the levels of agalactosyl IgG were significantly raised in IL-6 transgenic mice as reported earlier (Rook et al 1991a), the levels of β1,4-GalTase mRNA and enzyme activity did not show any significant difference between the transgenics and the control littermates. The data on β1,4-GalTase mRNA and enzyme activity levels in IL-6 transgenic mice were inconclusive (see section 6.3.1). It would have been useful to know the levels of IL-6 transgene in the mice used for the study of mRNA and enzyme activity levels. IL-6 did not show any significant effect on the level of β1,4-GalTase mRNA in human PBL cultures or on IgG galactosylation levels in human and murine cell cultures as measured by Bodman (1995). It is possible that higher concentrations of IL-6 may be needed to exert an effect on IgG galactosylation. It is also possible that other cytokines induced by IL-6 could play a role in reducing the level of IgG galactosylation in IL-6 transgenic mice. Examining the effect of only one
The role of the T cell in controlling IgG galactosylation was investigated in CD4-deficient MRL \( \text{Ipr/Ip r} \) mice. Although no significant difference in IgG galactosylation levels were detected between CD4-knockout mice and their heterozygous littermates, there was an indication (inconclusive due to the small number of samples) that the lack of CD4 prevented the rise in agalactosyl IgG with age. Experiments carried out by Rademacher \textit{et al} (1994) suggested that T cells are important in maintaining elevated levels of agalactosyl IgG, as the \%G\textsubscript{0} remained elevated longer when T-cell primed mice were used for the passive transfer of agalactosylated anti-collagen IgG into DBA/1 mice. Clearly, the role of the T cells in regulation of IgG galactosylation needs to be examined further.

Hormones have also been suggested to influence IgG glycosylation (Pekelharing \textit{et al} 1988, Rook \textit{et al} 1991b, Thompson \textit{et al} 1992) and \( \beta 1,4\)-GalTase levels (Rook \textit{et al} 1991b, Thompson \textit{et al} 1992). Haffar \textit{et al} (1988) found that glucocorticoids appear to regulate the trafficking of a subset of glycoproteins, but they do not affect the production of galactosylated glycoproteins, nor do they affect the specific activity of \( \beta 1,4\)-GalTase in Golgi vesicles. In our experiments, pregnancy (which is accompanied by hormonal changes) significantly altered the galactosylation status of serum IgG in Balb/c mice, but the levels of \( \beta 1,4\)-GalTase mRNA and activity in splenic lymphocytes were not affected. This was in spite of a dramatic rise in the expression of \( \beta 1,4\)-GalTase gene in the mammary tissue as reported previously by others (Harduin-Lepers \textit{et al} 1993). Prolactin, specifically, did not exhibit any effect on IgG galactosylation and showed only a minor effect on \( \beta 1,4\)-GalTase gene expression in human lymphocytes \textit{in vitro}.

The role of cellular activation on \( \beta 1,4\)-GalTase mRNA levels was also investigated. The mitogens PHA and PWM, the phorbol ester PMA, and the
ionophore ionomycin did not influence the β1,4-GalTase mRNA levels in human PBL cultures in our experiments. In support of these findings, Masibay et al (1991) did not detect any changes in expression of the β1,4-GalTase gene in cells stimulated by TPA (a phorbol ester) or ionophore A23187. Haffar et al (1988) also reported that ionophore treatment of hepatoma cells did not significantly affect either β1,4-GalTase activity or the galactosylation of glycoproteins. With regards to the effect of cAMP on β1,4-GalTase, the preliminary experiment described in this thesis and the study by Masibay et al (1991) did not detect significant changes on β1,4-GalTase mRNA and enzyme activity levels respectively. However, the finding of increased activity of β1,4-GalTase enzyme induced by forskolin in PC 12 cells (Roth et al 1991) and increased β1,4-GalTase mRNA levels due to retinoic acid and dibutyryl cAMP treatment of tetracarcinoma cells (Kudo and Narimatsu 1995), calls for more investigation on the long-term effect of cAMP on β1,4-GalTase in B cells.

In addition to the activity of the transferases, the rate of intracellular transport through the Golgi complex may influence the glycosylation reaction. It was shown by Wang et al (1991) that the poly-N-acetyllactosamines attached to lysosomal membrane glycoproteins are increased by the prolonged association with the Golgi complex caused by lowering the temperature at which the cells are incubated.

Changes in the biosynthesis and assembly of IgG polypeptides, as regards to the rate and timing of heavy and light chain association and formation of disulphide bonds may affect the exposure time of the glycoprotein to the terminal glycosyltransferases in the Golgi complex (Rademacher et al 1995). The order of chain assembly is reported to vary among the immunoglobulin classes and subclasses (Sutherland et al 1972). Studies on the preferred pathways of interchain disulphide formation for murine immunoglobulins have revealed that in the case of IgM and IgG2b, the heavy and light chains assemble before the two heavy chains are linked together to form the
complete molecule. In the case of IgG1, IgG2a, and IgA, two heavy chains are assembled before the light chains are linked to the disulfide bonded heavy chains to form the complete IgG molecule (Baumal et al 1971). Linking together of the two heavy chains later rather than sooner could make the Fc oligosaccharide more accessible for further glycosylation. Analysing the galactosylation status of polyclonal murine IgG isotypes has revealed that IgG1 exhibits the highest %G0 followed by IgG2a, IgG3, and IgG2b the lowest (William and Rademacher 1996). As the levels of IgG1 and IgG2a are increased in the sera from MRL lpr/lpr mice (Sobel et al 1991), this could be one explanation for increased levels of agalactosyl IgG observed in MRL lpr/lpr mice (Williams and Rademacher 1996) and IL-6 transgenic mice (as discussed earlier). Increased levels of agalactosyl IgG in patients with RA, however, has been shown to occur across all four IgG isotypes (Tsuchiya et al 1994, Rademacher et al 1995). If the proposed model of IgG chain assembly is correct, then in RA there is an increase in flux through the second pathway (i.e. the first interchain disulphide bond forming between the heavy chains) independent of the IgG subclass.

The conformation of IgG has specifically been shown to be important in controlling the oligosaccharide structure expressed on the IgG. Studies done in vitro using purified β1,4-GalTase from rat liver Golgi, bovine colostrum, and calf thymus have shown that β1,4-GalTase preferentially transfers galactose to the 1,3 branch of an acceptor complex biantennary oligosaccharide (Paquet et al 1984, Blanken et al 1984), including the oligosaccharide chain on denatured human IgG (Fujii et al 1990). However, in vivo the biantennary complex oligosaccharide of human and bisected bovine IgG is monogalactosylated on the 1-6 arm (Fujii et al 1990, Lund et al 1993) suggesting that the relative accessibility of the 1-3 arm and the 1-6 arm to β1,4-GalTase is altered when the oligosaccharide is covalently attached to a protein with which it can interact.
The influence of the polypeptide structure of the heavy chain in directing the action of transferases have been shown by analysing the glycosylation profiles of chimaeric antibodies produced in the same cell line. Using such an approach, Lund et al (1993) demonstrated different oligosaccharide profiles for each of the chimaeric mouse-human IgG1, IgG2, IgG3, and IgG4 antibodies produced in the J558L cell line. Using cloned hybrid myeloma cell lines (constructed by fusing two cell lines producing IgGs of different subclasses), Lee and colleagues (1990) analysed the glycosylation of two different IgGs and showed that they differed in the quantity and nature of terminal galactosylation. These studies indicated the importance of IgG structure rather than the transferases in influencing IgG glycosylation.

The work reported in this thesis clearly shows that the reduced IgG galactose levels in RA are not caused by decreased levels of β1,4-GalTase mRNA in B cells. It seems that expression of the β1,4-GalTase gene is tightly controlled. This is probably related to the fact that β1,4-GalTase is a house-keeping enzyme important for galactosylation of glycoproteins needed for the survival of the organism. The mechanism(s) of reduced IgG galactosylation in RA are, therefore, still to be fully elucidated. More generally, important functional roles for oligosaccharides on proteins are increasingly being appreciated. Understanding how the enzymes that control oligosaccharide synthesis are regulated is crucial to the future progress. Determining the effect of the three-dimensional structure of a protein on its glycosylation, and the biological relevance of microheterogeneity (glycoforms), remains a significant and exciting challenge.
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PUBLICATIONS


ABSTRACTS


IgG glycosylation in autoimmune-prone strains of mice

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SUMMARY
The relationship between increased levels of IgG oligosaccharide chains lacking galactose (G0) and the development of rheumatoid arthritis is unclear. In order to further our understanding of the observed correlation between raised serum G0 and arthritis, we have studied G0 levels in arthritis-prone and non-susceptible (i.e. non-arthritis-prone) mice and the effects on G0 of mycobacterial antigens, which have been postulated to play a role in the early events leading to the development of arthritis. We have shown that different age-matched mouse strains have characteristic 'resting' levels of G0 which (in six out of seven strains of mice) increase with age. We have also shown that these increases can be enhanced by immunization of arthritis-prone strains of mice with an adjuvant containing mycobacteria (Freund’s complete adjuvant (FCA)), suggesting that defects in the ability to regulate these G0 changes may be related to susceptibility to arthritis.

Keywords G0 autoimmunity mycobacterial antigens mice

INTRODUCTION
The percentage of IgG molecules lacking terminal galactose from the oligosaccharides on the Fc is raised in rheumatoid arthritis (RA) [1], and in some mycobacterial infections such as Crohn’s disease, tuberculosis [2] and leprosy [3]. This agalacto-syl glycoform (G0) also rises in normal human populations as a function of age [4,5].

G0 has also been shown to be elevated in spontaneous and induced murine models of arthritis. Thus, MRL Ipr Ipr (MRL Ipr) mice [6,7], CBA/J mice with pristane-induced arthritis [8] and male DBA/1 mice with collagen-induced arthritis [9] all show elevated levels of G0.

These circumstantial associations have led to the hypothesis that G0 plays a pathogenic role in the early stages of development of arthritis. There is some experimental evidence for this. Treatments that reduce or eliminate the early rise in G0 after an intraperitoneal injection of pristane result in a greatly reduced incidence and severity of arthritis at 150 days [8]. More direct evidence has come from the recent observation that murine antibodies to conformational epitopes on type II collagen have greatly enhanced ability to initiate arthritis after enzymatic removal of terminal galactose [10].

In this study we have investigated the possibility that serum IgG G0 may act as an indicator of susceptibility to spontaneous or induced forms of murine arthritis. We have followed G0 changes with time in a number of autoimmune-prone and non-autoimmune-prone strains of mice. Since immunization with Freund’s complete adjuvant (FCA) alone has been shown to be sufficient to produce arthritis in BALB/c mice [11], we have also studied the role of mycobacterial adjuvant in increasing the levels of G0 in arthritis-prone and non-susceptible strains of mice.

MATERIALS AND METHODS

Animals
Female CBA/Ca and BALB/c mice were purchased from the National Institute for Medical Research (Mill Hill, London, UK) and male DBA/1, DBA/2 and SWR mice from Harlan-OLAC Ltd (Bicester, UK).

Female C57Bl/6, AKR, A/J, SJL, NZW, NZB and male DBA/2 were also purchased from Harlan-OLAC Ltd and MRL +/+ and MRL Ipr were bred in our animal house facility. Non-obese diabetic strain (NOD) mice were obtained from the Clinical Research Centre (Harrow, London, UK).

Serum collection and storage
Blood from groups of five untreated female CBA/Ca, NZW, DBA/2, MRL +/+ , AKR, A/J, C57Bl/6, NZB, DBA/1, MRL Ipr, SJL and NOD mice was obtained from the retro-orbital sinus at 2 months of age and the sera stored at —20°C for G0 analysis.

In a separate experiment, groups of three to five untreated female CBA/Ca, MRL +/+ , MRL Ipr, NZW, NZB and male
DBA/1 mice were bled at 2, 3, 4, 5, 6 and 8 months of age and the sera stored as above.

Immunization protocols
Groups of four female CBA/Ca and BALB/c mice, aged 17 and 19 weeks respectively, were injected with 100 μl of PBS or a 1:1 emulsion of PBS:FCA (H37Ra strain, Difco Labs Ltd, East Molesey, UK). These mice were injected subcutaneously in the hind footpads (50 μl/foot) or at the base of the tail or intraperitoneally.

In a second study, groups of 5-13 male 10-week-old DBA/1, DBA/2 and 9-week-old SWR mice were immunized as above by subcutaneous injection into the hind footpads alone.

Sequential bleeds were taken over a period of 7-16 months and the resultant sera stored at −20°C until required.

Immun assay for G0
The level of serum G0 IgG was determined using modified immunoassays as described previously [5,12]. Briefly, 96-well Maxisorb-immunoplates (Life Technologies Ltd, Paisley, UK) were coated with 50 μl well protein A (Sigma Chemical Co., Poole, UK) at 2.5 μg/ml in PBS and incubated overnight at 4°C. The wells were aspirated and blocked with PBS-1% bovine serum albumin (BSA) containing 0.05% Tween 20 (PBS-TBSA) for 1 h at 37°C followed by three washes with PBS-TWEEN. Samples and standards with known G0 levels were diluted 1:50 in buffer consisting of 0.1 M glycine and 0.16 M NaCl adjusted to pH 8.0 with 0.1 M NaOH.

Aliquots of diluted sera (50 μl) were added in triplicate to two identical plates and incubated for 2 h at 37°C. After washing, 50 μl well PBS were added and the plates floated on a waterbath at 85°C for 15 min to partly denature the IgG molecules exposing the oligosaccharides. The biotinylated lectin Bandeiraea simplicifolia (Vector Labs, Breton, UK), containing 0.1 mM CaCl₂, was diluted 1:50 (20 μg/ml) and biotinylated sheep F(ab)₂ anti-mouse IgG (Sigma) 1:20000 (0.05 μg/ml) in PBS-TBSA and each added at 50 μl/well to one of the cooled duplicate plates and incubated at 4°C overnight. After three washes, 50 μl well of streptavidin-peroxi-dase (Dako Ltd, High Wycombe, UK) was added and incubated at 37°C for 1 h. A colour reaction was produced using 50 μl well 0.1 M citrate phosphate buffer pH 4.1 containing 0.5 mg ml 2.2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (Sigma) and 1:2000 hydrogen peroxide. After 15 min the reaction was stopped with 50 μl well NaF (2 mg/ml) and the plates read on an automated ELISA reader (Dynatech Labs, Billinghurst, UK) at 410 nm.

The results were expressed as Bandeiraea optical density anti-IgG optical density and sample G0 levels extrapolated from the curve of mouse standards with known G0 values (as determined by the biochemical method [13]) from the respective plates.

Statistical analysis
Mean G0 levels (± s.e.m.) were plotted for all groups of mice. Simple curve fit analysis was performed on the G0 values of the untreated strains of mice with time, and analysis of variance (ANOVAR) was performed on the mean area under the curve for each group of PBS, FCA emulsion or PBS immunized mice over time.

RESULTS
G0 levels in autoimmune and non-autoimmune-prone strains
The G0 levels of serum IgG from 12 strains of mice at 2 months of age were analysed. The mean levels of G0 varied widely between strains, ranging from 20.6±1.51 to 55.2±2.51 (mean ± s.e.m.), depending on the strain of mouse studied (Fig. 1).

Age-related changes in G0
G0 levels were analysed in serum IgG from seven strains of mice and plotted against age (Fig. 2). A significant increase in the level of G0 was seen in each of the strains studied over time, with the exception of NZB mice, in which increased levels up to 4 months of age, apparently followed by a decrease, were seen.

Increase of G0 levels by FCA
Administration of FCA resulted in a significant increase in serum G0 compared with PBS-injected mice in the arthritis-prone BALB/c strain, whereas non-susceptible control groups of CBA/Ca mice showed no significant difference in G0 between PBS and FCA immunization (Fig. 3). There was no significant difference between route of FCA administration over the entire time period studied, as determined by ANOVAR of the areas under the curve (Fig. 4).

In the second study, as above, the arthritis-prone DBA/1 FCA-injected mice showed significantly higher levels of G0 than the control PBS-injected mice throughout the investigation.
Autoimmune mice and G0

Table 1. The areas under the curve of G0 change with time for PBS or PBS: Freund’s complete adjuvant (FCA) emulsion-injected groups of mice. (Susceptible DBA/1 n = 5-13, DBA/2 n = 5 and SWR n = 5) are shown for early and late phase of the experiment.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>Early (day 0-112)</th>
<th>Late (day 112-217)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible DBA/1</td>
<td>PBS</td>
<td>2666±0</td>
<td>* 3298±1</td>
</tr>
<tr>
<td>Non-susceptible SWR</td>
<td>PBS</td>
<td>3339±4</td>
<td>* 3974±3</td>
</tr>
<tr>
<td>Non-susceptible DBA/2</td>
<td>PBS</td>
<td>2509±3</td>
<td>NS 2983±1</td>
</tr>
<tr>
<td>PBS: FCA</td>
<td>2977±6</td>
<td></td>
<td>3642±2</td>
</tr>
<tr>
<td>PBS: FCA</td>
<td>3044±0</td>
<td></td>
<td>3708±6</td>
</tr>
<tr>
<td>PBS: FCA</td>
<td>2588±4</td>
<td>NS</td>
<td>3207±7</td>
</tr>
</tbody>
</table>

NS, No significant statistical difference; * significant statistical difference (P<0.01) after analysis of variance (ANOVAR) of these areas.

DISCUSSION

In this study we have shown that different mouse strains have characteristic ‘early’ levels of serum G0 which increase with age and are enhanced by immunization with FCA in the arthritis-prone strains.

The resting level of G0 in 2-month-old mice from 12 autoimmune-prone and non-autoimmune-prone strains varied widely (Fig. 1). No correlation with susceptibility to induced autoimmunity was evident, but of the three strains developing spontaneous autoimmune disease (lupus-like syndrome and an arthritis in MRL Ipr [14], diabetes and polyendocrine disease in NOD [15,16] and haemolytic anaemia in NZB [17]), the NOD and MRL Ipr had far and away the highest G0 values (55.2±2.52 and 44.4±0.8 respectively). The third strain, NZB was, however, unusual, with relatively low levels of G0 at 2 months of age, rapidly increasing to 55.7±2.48 by 4 months of age (Fig. 2), concurrent with the spontaneous onset of their Coombs’ positivity [17]. The possible pathogenic role of the G0 antibody to band 3 of the erythrocyte antigen in NZB mice [18] is currently under study. Autoimmune-prone strains such as DBA/1 [19], SJL [20,21] and MRL + /- Ipr [22] with lower resting levels of G0 than MRL Ipr and NOD mice, still develop autoimmune disease later in life, but this is often only after induction by various agents.

These findings indicate that G0 alone cannot be used as an indication of the development of autoimmune diseases in these strains. This is in contrast with human RA, in that early onset synovitis patients before developing RA, have raised levels of G0 compared with those individuals who do not go on to develop the disease, indicating a predictive value for increased IgG G0 [23,24].

An increase in the level of G0 in the different mouse strains occurred with age, closely paralleling the changes seen in human populations [2,3]. In this study the G0 levels were only determined up to the age of 8 months. It is not clear whether G0 would continue to rise, plateau or indeed fall (as seen by the results of the NZB mice). Nor was it possible to study the levels soon after birth of the mice, which in man were found to be higher than at 25 years of age.

Although the heating step in the G0 assay, which is essential to expose the Fc-associated oligosaccharides, leads to the release
of some IgG from protein A, there is equal loss of all IgG subclasses (T. Rademacher, personal communication). These age-related changes are therefore unlikely to be due to selection of a specific subclass of IgG showing elevated G0 levels and increasing in relative concentration with age. The elevated G0 level could be related to the increased spontaneous release of IL-6 which occurs in ageing animals [25,26]. This cytokine is known to be associated with increased galactosylation, since raised G0 was seen in transgenic mice over-expressing the human IL-6 gene, and serum G0 rises following injection of recombinant IL-6 [12]. It is not known whether similar age-related increases in IL-6 release occur in man.

Some of the diseases in which strikingly raised G0 is found, such as leprosy and tuberculosis, are known to be caused by mycobacteria [3,27]. Others such as Crohn’s disease [28] and sarcoidosis [29] are associated with the presence in the lesions of mycobacterial genomic material in at least a percentage of cases [30]. Immunization of arthritis-prone female BALB/c and male DBA/1 mice with one dose of FCA caused a significant increase in the serum G0 compared with those mice injected with PBS alone, but did not do so in two of the control strains (female CBA/Ca and male DBA/2 mice) (Fig. 3 and Table 1). These data are in agreement with similar published observations that exposure to mycobacteria results in increased serum G0 [31].

The control non-susceptible strain SWR showed early significant differences in G0 between groups of PBS and FCA-injected mice, but seemed to be able to correct and regulate the levels in the latter phase of the study (Table 1). This was seen to a lesser extent in the FCA-injected non-susceptible CBA/Ca mice, which although they did not develop a significant long-term increase in G0, showed a tendency for increased G0 early after injection which then levelled out with time (Fig. 3). This suggests that the arthritis-prone mice fail to regulate the level of G0, which together with another event (such as the autoantibody production in collagen arthritis [10]) could precipitate the disease.

It has been shown that DBA/1 males develop spontaneous arthritis at 5 months of age [32] and that BALB/c female develop histological features of arthritis after FCA immunization at a similar time point [11]. None of the arthritis-prone mice in this study, however, showed any signs of development of arthritis. This may be due to the fact that none of the mice were immunized intradermally (the route shown to be most effective for the induction of collagen arthritis [33]). Other considerations include the fact that the same strain of mice may behave differently in different animal facilities (as seen in the induction of 16/6 idiotype lupus model in BALB/c mice [34]), the mycobacteria in the FCA used in our study was H37Ra (different from that used in [11]), and the mice were not boosted. From these experiments and those reported by others, it seems possible that both an arthritogen and FCA are required via an arthriogen route (intradermal) in order to precipitate a quick onset of arthritis and a rapid increase in G0 levels, whereas, FCA alone intradermally produces a later onset of arthritis [11] and FCA alone by other routes a slower increase in G0 levels.

It is interesting that the early (day 0-40) fluctuations in serum G0 following immunization were seen not only in BALB/c and CBA/Ca mice injected with FCA, but also with PBS (Fig. 3). This would suggest that ‘immunization stress’ alone can modify IgG G0. Such changes have been seen following immunization of mice with pristane [12], which the authors suggest could be due to bursts of IL-6 activity. This would also support the idea that early events in the response to ‘arthritogens’ are critical for the course of the development of the disease. In this regard, it has been reported (but not explained) that RA is occasionally precipitated by stress [35] and that male DBA/1 mice, if kept in non-aggressive conditions (less than three mice per cage), do not develop spontaneous arthritis [36].

The role of G0 in experimental models is unclear at present, but is likely to be one of the factors which contribute to the development of arthritis [10]. Using these models, our continuing studies will address the question as to the site of G0 IgG production and its regulation by IL-6 and other cytokines at the cellular level.

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Autoimmune mice and GO


Reduced galactosyltransferase mRNA levels are associated with the agalactosyl IgG found in arthritis-prone MRL-lpr/lpr strain mice

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SUMMARY
MRL-lpr/lpr strain mice have defectively glycosylated IgG. This may be related to the rheumatoid arthritis (RA)-like disease that occurs in these mice, because a similar glycosylation defect is seen in human subjects with RA. Whilst it is known that this defect is associated with reduced activity of the β-1,4-galactosyltransferase (β-1,4-GalTase) enzyme, the cause of this reduced activity is at present unknown. We have therefore examined the molecular genetics of β-1,4-GalTase in MRL-lpr/lpr mice. Using 10 different restriction endonucleases we found no evidence for a polymorphic variant of the gene in glycosylation-defective mice. However, the level of mRNA for β-1,4-GalTase was lowest in the MRL-lpr/lpr strain mice, the strain with the most poorly galactosylated IgG of the four strains examined. Thus, the reduced level of IgG oligosaccharide galactosylation found in MRL-lpr/lpr strain mice appears to be related to either an altered transcriptional level of, or altered mRNA stability for, β-1,4-GalTase in lymphocytes from these mice.

INTRODUCTION
Approximately 30 different complex-type N-linked biantennary oligosaccharide structures have been found on IgG and are predominately located at asparagine 297 in the C2 domain of the Fc region. These sugar chains are thought to play a crucial role in maintaining the three-dimensional structure of the Fc portion of IgG, and depletion of the entire sugar moiety from the Fc has been shown to result in a decrease of some of the biological activities of IgG. Disease-associated defects in IgG glycosylation were initially documented in rheumatoid arthritis (RA), a disease in which patients were found to have reduced levels of galactose on the IgG oligosaccharides. Subsequent investigations of IgG galactose levels showed that this defect was also present in a highly restricted number of clinical entities, including Crohn’s disease, tuberculosis and systemic lupus erythematosus (SLE) associated with Sjögren’s syndrome. IgG which bears agalactosyl oligosaccharides has a functional impairment in its ability to bind to cellular Fc receptors and to C1q. Furthermore, recent evidence indicates that the agalactosyl glycoform of IgG autoantibodies are pathogenic in type II collagen-induced arthritis in mice and are a relatively important prognostic marker for human RA.

Protein glycosylation is mediated by intracellular processing enzymes, including N-acetylglucosamine β-1,4 galactosyltransferase (β-1,4-GalTase), which specifically transfers galactose from UDP-galactose onto acceptor sugars containing terminal non-reducing N-acetylglucosamine (GlcNAc). The reduced galactosylation of IgG in RA is seen on newly secreted IgG and is associated with decreased lymphocytic β-1,4-GalTase activity. The glycosylation defect in this disease would therefore appear to be due to an intrinsic cellular abnormality, rather than due to a post-secretory degradative process.

MRL-lpr/lpr mice spontaneously develop an autoimmune disease similar to human RA in that they possess circulating rheumatoid factors and immune complexes, and develop joint inflammation. It has recently been shown that the parallel with human disease extends to the IgG glycosylation defect both with regard to decreased IgG galactose levels and reduced B-cell β-1,4-GalTase activity. In order to help define the basis of this defect in the MRL-lpr/lpr mouse, we have investigated the level of β-1,4-GalTase mRNA expression and IgG galactosylation in these mice.

MATERIALS AND METHODS
Mice
NOD strain mice were obtained from the Clinical Research Centre (Harlow, UK). All other strains of mice were purchased at 7 weeks of age from Harlan Olac Ltd (Bicester, UK) and then...
maintained in the UCL animal facility. All the mice used in this study were 3–5-month-old females. Retro-orbital bleeds were taken at desired time-points and mice were later killed by cervical dislocation prior to removal of spleens.

**IgG galactosylation**

The percentage of serum IgG oligosaccharide chains lacking galactose was determined using a previously described protein capture immunoassay with the lectin *Banderaea simplicifolia* (Vector Labs, Peterborough, UK) and sheep F(ab')2 antigoat IgG antibody (Sigma, Poole, UK) to detect the ratio of terminal N-acetylglucosamine-IgG. The results were then extrapolated from a standard curve of murine IgG with known galactose contents run with each test.

**Southern blotting assays**

DNA was extracted from freshly isolated liver by proteinase K digestion and phenol–chloroform extraction. Ten micrograms of genomic DNA was digested to completion with 25–50 U restriction endonuclease. Restriction fragments were separated by electrophoresis on 0.6% agarose gels and, following partial hydrolysis in 0.125 M HCl for 20 min, denaturation in 0.5 M NaOH, 1.5 M NaCl for 40 min and neutralization in 1.5 M NaCl, 5 M Tris, pH 8.0, for 40 min, transferred to Hybond-N nylon membranes (Amersham International, Amersham, UK). DNA was cross-linked to the membrane using a UV Stratalinkr (Stratagene, La Jolla, CA).

Blots were prehybridized for a minimum of 2 hr at 65° in hybridization buffer (450 mM sodium chloride, 45 mM sodium citrate, pH 6.15, 0.2% Ficoll, 0.2% BSA fraction V, 0.2% polyvinylpyrrolidone, 0.1% SDS, 10% dextran sulphate and 0 µl/ml of denatured salmon sperm DNA) followed by hybridization at 65° overnight. The 32P-labelled murine β-1,4-GalTase cDNA probe is approximately 2.3 kb in size and contains 1.6 kb of murine β-1,4-GalTase cDNA, corresponding to nucleotides 241–1884 of the published sequence. Final washing conditions were 45 mM sodium chloride, 45 mM sodium citrate, pH 6.15, and 0.1% SDS at 65°.

**Isolation and preparation of RNA**

Pleural cell suspensions were prepared in RPMI-1640 medium with 5% fetal calf serum (FCS). After lysing the red cells, macrophages were removed by adherence to plastic at 37° for 1 hr, following which non-adherent cells were washed in PBS prior to RNA isolation using the single-step method of acid guanidium thiocyanate–phenol–chloroform extraction.

**Ribonuclease protection assays**

A 1 kb HindIII–HincII fragment containing nucleotides 241–163 of murine β-1,4-GalTase was subcloned into pBluescript K (Stratagene). The remaining 400 bp is a vector sequence on the pCDM8 (Invitrogen, San Diego, CA) vector utilized during the original cDNA isolation. The plasmid was linearized with SstI (nucleotide 704) and a 32P-labelled complementary oligo-stranded RNA probe made to a specific activity of approximately 107 c.p.m./µg using T7 RNA polymerase according to manufacturer's instructions (Stratagene). This probe thus corresponds to nucleotides 704–1263 (from SstI to BglII) of the murine β-1,4-GalTase sequence and contains no promoter-derived sequences. The murine β-actin probe (Ambion, Austin, TX) was synthesized to a purposefully lower specific activity of approximately 105 c.p.m./µg and the probes purified using PAGE. The ribonuclease protection assay (RPA) was performed using the RPA II kit (Ambion) following procedures recommended by the manufacturer. Briefly, an excess of both of the labelled cRNA probes was mixed with 10 µg of sample RNA and incubated at 42° for at least 16 hr to allow hybridization of the probes to target mRNA contained within the sample RNA. The mixture was then treated with 0.1 U RNase A and 20 U RNase T1 for 30 min at 37°. Protected fragments were analysed on a 5% polyacrylamide–8 M urea gel.

The level of β-1,4-GalTase mRNA and of steady-state β-actin mRNA was assessed by scanning densitometry of autoradiographs from preflashed Fuji XR film using a Bio-Rad 600 densitometer (Bio-Rad, Richmond, CA), and by liquid scintillation counting in Aquasol (New England Nuclear, Du Pont, Stevenage, UK) of the radioactivity in the bands excised from the gel (using a Minaxi TriCarb 4000 counter; Packard, Downers Grove, IL). Results are expressed as the ratio of β-1,4-GalTase:actin mRNA.

**RESULTS**

The β-1,4-GalTase gene has a similar structure in each of 11 strains of inbred mice

Using a panel of 10 restriction endonucleases (BanHI, BglII, EcoRI, HincII, HindIII, MspI, PvuII, KsaI, SacI and TaqI) no polymorphisms were detected for the gene encoding β-1,4-GalTase in any of 11 strains of autoimmune-prone and non-autoimmune-prone mice (AJ, AKR, C57Bl/6, CBA/Ca, DBA/2, MRL-+/+, MRL-lpr/lpr, NOD, NZB, NZW and SJL) (data not shown). Thus, this gene locus would appear to have a similar organization in all the murine strains examined, including the MRL-lpr/lpr strain which shows reduced IgG galactose levels.

**Reduced levels of β-1,4-GalTase mRNA in MRL-lpr/lpr strain mice**

Lymphocytic β-1,4-GalTase mRNA levels were measured in four different strains of mice (MRL-lpr/lpr, MRL-+/+ and CBA). The results of an experiment comparing MRL-lpr/lpr with CBA/Ca strain mice are shown in Fig. 1. Visually, differences were seen most obviously when lanes with comparable levels of loading, such as MRL-lpr/lpr lane 3b and CBA/Ca lane 3a, were compared. In order to compensate for different amounts of RNA between lanes, results were quantified as the ratio of the signal (peak area in the case of densitometric analysis and c.p.m. for scintillation measurements) obtained for β-1,4-GalTase mRNA over β-actin mRNA. The values obtained by both methods were lower for all three 5-month-old female MRL-lpr/lpr mice compared to those for age- and sex-matched CBA/Ca mice (Table 1; P = 0.002, unpaired Student's t-test), indicating a reduced level of β-1,4-GalTase mRNA expression relative to β-actin mRNA expression in MRL-lpr/lpr compared to CBA/Ca strain mice.

The data obtained from five separate experiments comparing the levels of β-1,4-GalTase mRNA between four different strains are shown in Fig. 2. Unlike MRL-lpr/lpr mice, the genetically similar MRL-+/+ mice had levels of β-1,4-GalTase mRNA that were comparable to those seen in CBA/Ca strain.
IgG glycosylation in the different strains of mice

Levels of IgG galactose, derived from a standard curve of IgG of known galactose content, were measured in sera obtained from mice at the same time as the spleens were removed. The percentage of chains lacking galactose was greatest in MRL-lpr/lpr strain mice. The reduced level of β-1,4-GalTase mRNA seen in splenic lymphocytes from the MRL-lpr/lpr mice, therefore, correlated ($r = 0.86, P = 0.026$) with the increased level of exposed IgG GlcNAc in the same mice (Fig. 3). In one of the six MRL-lpr/lpr mice there was a higher level of β-1,4-GalTase message, and this was associated with higher levels of IgG galactose. Although the SJL strain mice showed lower levels of β-1,4-GalTase mRNA than MRL-+/+ and CBA/Ca, the reduction in mRNA levels was not as profound as in the MRL-lpr/lpr mice, and was not significantly associated with reduced levels of galactose on serum IgG.

**DISCUSSION**

Abnormal galactosylation of IgG in arthritis-prone MRL-lpr/lpr strain mice parallels a similar, well-established abnormality in human RA. As in human subjects, the amount of galactose on murine IgG oligosaccharides varies with the age of the mice. Decreased levels of lymphocytic β-1,4-GalTase activity are found in MRL-lpr/lpr mice compared to CBA/Ca strain mice. Taken together with the fact that normal galactosylation is re-established during disease remission in patients with RA, the results of the restriction fragment length polymorphism analysis we have carried out would argue against a defective allelic variant of the β-1,4-GalTase gene being associated with the reduced IgG galactosylation seen in these diseases.

The reduced steady-state levels of β-1,4-GalTase mRNA we have observed in splenic lymphocytes from MRL-lpr/lpr strain mice.

**Figure 2.** β-1,4-GalTase/β-actin mRNA levels in splenic lymphocytes from four strains of mice. The levels from five separate experiments are normalized against the mean value (arbitrarily assigned a value of 100) obtained in each experiment for the CBA/Ca strain.

**Figure 3.** Correlation between percentage serum IgG lacking galactose and β-1,4-GalTase mRNA levels. (□) MRL-lpr/lpr; (■) SJL; (▲) MRL-+/+; (○) CBA/Ca. Note that although 10 CBA/Ca mice are included in the study, they appear as a single data point (i.e. 100 versus 100) against which the other strains have been normalized.

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**Table 1.** Quantification by two different methods of the RPA results from the experiment presented in Fig. 1

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The values given are the ratio of β-1,4-GalTase mRNA signal over β-actin mRNA signal.
Recent evidence in the collagen type II murine model of RA indicates a pathogenic role for abnormally galactosylated IgG. It will be of interest to examine the effect of glycosylation-modifying cytokines and other soluble mediators on galactosyltransferase gene expression and disease in MRL-lpr/lpr mice. This may help provide clues as to the cause of the reduced β-1,4-GalTase mRNA levels and of defective IgG galactosylation in these mice, perhaps ultimately allowing correction of the glycosylation defect in autoimmune rheumatic diseases.

ACKNOWLEDGMENTS

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Agalactosyl IgG and $\beta$-1,4-galactosyltransferase gene expression in rheumatoid arthritis patients and in the arthritis-prone MRL
$lpr/lpr$ mouse

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SUMMARY

Reduced galactosylation of immunoglobulin G (IgG) is well documented in rheumatoid arthritis (RA), but the reason for this defect is still unknown. There is some evidence supporting a defect in the biosynthetic pathway, and a reduction in the level of $\beta$-1,4-galactosyltransferase ($\beta$-1,4-GalTase) enzyme activity. Since glycosyltransferases are, in general, regulated at the level of transcription, we have measured the level of $\beta$-1,4-GalTase gene expression in B cells from patients with RA and normal control individuals. We found no significant difference in mRNA levels for the transferase in these two groups ($P > 0.7$). MRL/Mp-lpr/lpr (MRL-lpr) mice develop a spontaneous arthritis with increased levels of agalactosyl IgG (G0). In spite of a significant reduction in the level of $\beta$-1,4-GalTase mRNA in total spleen lymphocytes from MRL-lpr mice compared with the congenic MRL/Mp-+/-+ (MRL-+/-+) mice and with CBA/Ca mice, we found comparable levels of the $\beta$-1,4-GalTase mRNA in purified B cells from both spleen and lymph nodes of the three strains. Amongst the lymphoid compartments examined, the spleen and peripheral blood were found to be the major contributors of G0 in MRL-lpr mice. These data indicate that in neither human RA, nor in an animal model of this disease, is reduced IgG galactosylation caused by impaired expression of the $\beta$-1,4-GalTase gene in B lymphocytes. Furthermore, splenic B cells, which have normal levels of $\beta$-1,4-GalTase mRNA, appear to be a major source of G0 in MRL-lpr mice.

INTRODUCTION

Rheumatoid arthritis (RA) is associated with defective galactosylation of Immunoglobulin G (IgG). When compared with age-matched controls, patients have reduced galactose levels of the oligosaccharide chains linked at asp 297 of the C2 domain. Agalactosyl IgG (G0) shows significantly reduced binding to Clq and to Fc receptors. Multiple presentation of IgG G0 to mannose-binding protein (MBP) has been shown recently to result in activation of complement. In addition agalactosyl IgG is associated with pathogenicity in murine type II collagen-induced arthritis, and an elevated percentage of serum agalactosyl IgG is an important prognostic marker for human RA.

It has been suggested that defective galactosylation of IgG may be due to aberrant control of lymphocytic $\beta$-1,4-galactosyltransferase ($\beta$-1,4-GalTase) activity. This enzyme exerts several biological functions. First, as a Golgi-membrane bound enzyme, $\beta$-1,4-GalTase participates in the synthesis of oligosaccharide chains by specifically transferring galactose from an activated UDP-galactose donor to acceptor sugars containing terminal non-reducing N-acetylgalcosamine (GlcNac). Second, during pregnancy, the specificity of $\beta$-1,4-GalTase in lactating mammary gland is altered by binding to a modifier subunit, a-lactalbumin, to catalyse the synthesis of lactose. Finally, on the cell surface, $\beta$-1,4-GalTase is thought to function as a recognition molecule during a variety of cell–cell and cell–matrix interactions by recognizing specific glycoconjugates on the cell surface and in the extracellular matrix. Thus, the role of $\beta$-1,4-GalTase has been documented in fertilization, differentiation and proliferation.

A single gene encoding $\beta$-1,4-GalTase has been localized to human chromosome 9 at band p13 and to mouse chromosome...
The gene locus spans >50 Kb of genomic DNA and is comprised of six exons. The β-1,4-GalTase locus has been shown to have two transcription initiation sites and therefore to specify two sets of mRNA transcripts encoding two forms of the protein which have a 13 amino acid difference in the length of the amino terminal domain. The gene is under the control of multiple promoters with the promoter controlling the first initiation site lacking in the smaller transcript. There appears to be separate promoters for housekeeping, mammary cell-specific, and germ cell-specific expression of β-1,4-GalTase.

MRL/Mp-lpr/lpr (MRL-lpr) mice spontaneously develop an autoimmune disease with some features that resemble human RA including the decreased IgG galactose level. Reduced B-lymphocyte β-1,4-GalTase activity has been reported in this arthritis-prone strain of mouse. We have shown previously that the level of β-1,4-GalTase mRNA is reduced in spleen cells from MRL-lpr mice compared with that from the normally galactosylated CBA/Ca strain mice, suggesting an association between the IgG galactose levels and β-1,4-GalTase gene expression. Now, we have extended our studies to the purified B cells from MRL-lpr mice and from patients with rheumatoid arthritis in order to define more closely the basis of the decreased IgG galactosylation and have also looked at the distribution of de novo IgG G0 secretion amongst the lymphoid compartments in MRL-lpr mice to see which ones are contributing to the high level of agalactosyl IgG found in the sera of these mice.

MATERIALS AND METHODS

Study subjects
Blood samples were obtained from patients with RA, each of whom fulfilled the American Rheumatism Association (ARA) revised criteria for the disease. The control population was composed of healthy volunteers.

Mice
Mice were purchased at 7 weeks of age from Harlan Olac Ltd (Bicester, UK) and then maintained in our local animal facility. All the mice used for mRNA analysis were 12-week-old females. Cell suspensions were prepared from spleen and lymph nodes, from pools of seven mice. The mice used to study the distribution of G0-secreting cells comprised pools of MRL-lpr mice (mean age 4 months, range 3–5 months), and CBA/Ca mice (mean age 5 months, range 3–7 months).

Serum IgG galactosylation
Serum IgG G0 in the human and murine individuals was measured as follows using a modified version of previously published assays. Ninety-six well maxisorb-immunoplates (Nunc, Roskilde, Denmark) were coated overnight with 50 μl/well of recombiant truncated protein G' [Sigma (Poole, UK), P-4689] at 50 μg/ml in phosphate-buffered saline (PBS) at 4°C. The wells were aspirated and blocked with 100 μl 0.05% Tween 20, 1% BSA in PBS (PBS-T-BSA) for 1 hr at 37°C followed by three washes with 0.05% Tween 20 in PBS. Standards with known G0 levels and sera diluted 1:100 (1:50 for murine) in 0.1 M glycine, 0.16 M NaCl, pH 7.0 were added in triplicate (50 μl/well) to two identical plates and incubated for 2 hr at 37°C. After washing, 50 μl/well PBS was added and the plates floated on a waterbath at 85°C for 15 min to partially denature the IgG molecules and thus expose the oligosaccharides. The biotinylated lectin Bandeiraea simplicifolia II (BSII, Vector Laboratories Inc., Cambridgehire, UK) at 0.4 μg/ml in PBS-T-BSA containing 0.1 mM calcium chloride, or biotinylated goat F(ab')2 anti-human IgG [or sheep F(ab')2 anti-mouse IgG (Sigma)] at 4 μg/ml in PBS-T-BSA, were added at 50 μl/well to the cooled plates and incubated at 4°C overnight. After three washes, 50 μl/well of Streptavidin-horseradish-peroxidase (DAKO Ltd., Buckinghamshire, UK) was added and incubated at 37°C for 1 hr. A colour reaction was produced using 50 μl/well 0.1 M citrate phosphate buffer pH 4.1 containing 0.5 mg/ml 2,2-azino-bis(3-ethylbenzhiazoline-6-sulfphonic acid) (Sigma) and 1:2000 hydrogen peroxide. After 15 min, the reaction was stopped with 50 μl/well sodium fluoride (2 mg/ml) and the plates were read on an automated enzyme-linked immunosorbent assay (ELISA) reader (Dynatech Laboratories Ltd, Billinghamurst, West Sussex, UK) at 410 nm. The results were expressed as the ratio of BSII:anti-IgG binding and quantified against the standard curve of known G0 samples. The G0 standards used in this study were human (both RA and control subjects) and murine (BALB/c, CBA/Ca, DBA/1 and MRL-lpr) IgG molecules whose G0 values were defined by comparison in both dot-blot and ELISA systems with human and murine IgG whose G0, G1 and G2 values had been determined by Dr. T. Rademacher and colleagues at the Department of Biochemistry, University of Oxford, using the hydrazinolysis method.

Murine cell cultures
Cell suspensions were prepared by disaggregation of bone marrow, lymph node and spleen from pools of mice. Erythrocytes were removed from all cell suspensions using 0.16 M ammonium chloride pH 7.2. The cells were washed twice before culture. Peripheral blood mononuclear cells were separated by centrifugation on Lymphopaqe (density 1.086 g/ml) at 750 g for 30 min at room temperature. The interface cells were washed twice prior to use. Cells were cultured for 4 days at 10⁶ cells/ml in complete medium consisting of: RPMI-1640 supplemented with 5% γ-globulin free, heat-inactivated fetal calf serum (FCS) (Life Technologies, Renfrewshire, UK), 2 mM L-glutamine, 50 U/mL penicillin-streptomycin, 50 mM 2-mercaptoethanol, 1 mM sodium pyruvate and 1% non-essential amino acids (Life Technologies, Renfrewshire, UK). The percentage of spontaneously secreted G0 was measured as above using BSII and Ricinus Communis agglutinin I, and IgG concentration was measured with an ELISA assay according to Shields et al.

Preparation of cells for mRNA measurements
Human peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over Lymphoprep (Nycomed, Oslo, Norway) at 800 g for 20 min at 18°C. Cells were removed from the interface and washed twice in Hanks' balanced salt solution (HBSS). To isolate peripheral blood lymphocytes, PBMCs obtained as above were resuspended at 5 x 10⁶/ml in RPMI-1640 containing 20% FCS, monocytes depleted by plastic adherence at 37°C for 1 hr, and the non-adherent cells washed twice in PBS prior to RNA extraction. Purified B cells were obtained by positive selection from PBMCs using anti-CD19 coated Dynabeads M-450 (Dynal, Oslo, Norway) following...
the manufacturer’s instructions. This population comprised >95% CD19+ cells in each experiment.

The B cells from the mouse spleen and lymph node cell suspensions were isolated using magnetic beads coated with anti-mouse IgG antibodies (Dynal), in accordance with the manufacturer’s instructions. This population consisted of >95% slgG+ cells in each experiment. The surface-Ig negative population was obtained by two rounds of depletion using anti-mouse IgG-coated Dynabeads. These cells were then placed on plastic at 37° for 1 hr to remove adherent cells, following which the non-adherent cells were washed twice in PBS prior to RNA isolation. This cell population consisted of >70% CD3+, <10% Mac-1- and <1% slgG+ in the case of spleen, and >85% CD3+, <2% Mac-1-, and <1% slgG+ cells in the case of lymph node.

Production of antisense RNA probes
The β-1,4-GalTase probe used for the human studies was prepared by subcloning a 150 bp SalI–PstI fragment containing nucleotides 58–208 of human β-1,4-GalTase exon 214 into pBluescript KS (Stratagene, La Jolla, CA). High specific-activity RNA probe (approximately 10⁷ c.p.m. /μg) was transcribed with T3 RNA polymerase (Stratagene) after linearization with PstI. The probe was 377-nucleotides long and the fragment protected by β-1,4-GalTase mRNA was 150-nucleotides long. The human β-tubulin cDNA clone Db-1 was used as an internal control to normalize for the amount of RNA in each sample lane. High specific-activity RNA probe (approximately 10⁷ c.p.m. /μg) was transcribed with T3 RNA polymerase from a 264 bp PstI fragment (nucleotides 128–392 of Db-1 cDNA clone25) subcloned into pBluescript KS (Stratagene) and linearized with NcoI. The probe was 232-nucleotides long and the fragment protected by β-tubulin mRNA 172-nucleotides long. pT7 RNA 18S antisense control template (Ambion, Austin, TX), also used as an internal control for the RPA, produced a 109-nucleotide run-off transcript, 80 nucleotides of which were complementary to human 18S ribosomal RNA. An 18S ribosomal RNA probe was made to a purposefully lower specific activity of approximately 10⁵ c.p.m. /μg. Following transcription and DNAse digestion, samples were extracted with phenol-chloroform and purified by polyacrylamide-urea gel electrophoresis.

The probes used for the studies on mice have been described previously. The probe recognizing murine β-1,4-GalTase (specific activity of approximately 10⁷ c.p.m. /μg) corresponds to nucleotides 704–1263 of the sequence published by Shaper et al.13 The murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (Ambion, Austin, TX), used as an internal control to normalize for the total amount of RNA, was synthesized to a purposefully lower specific activity of approximately 10⁶ c.p.m. /μg.

Ribonuclease protection assays (RPA)
Total cellular RNA was isolated by acid guanidium thiocyanate-phenol-chloroform extraction.27 The RPA was performed using the RPA II kit (Ambion) according to the manufacturer’s instructions. Briefly, an excess of both the β-1,4-GalTase and control-labelled RNA samples were mixed with sample RNA and incubated at 42° overnight to allow hybridization of the probes to target mRNA contained within the sample RNA. The mixture was then treated with 0-1U RNase A and 20U RNase T1 for 30 min at 37°. Protected fragments were analysed on a polyacrylamide-urea gel. The level of β-1,4-GalTase mRNA and of steady-state control mRNA was assessed either by scanning densitometry of autoradiographs from pre-flashed Fuji XR film using a Bio-Rad GS-250 Molecular Imager (Bio-Rad, Richmond, CA), or by scanning the gels using a Bio-Rad GS-250 PhosphoImager. Results are expressed as the ratio of β-1,4-GalTase mRNA: control signal.

RESULTS
Levels of β-1,4-GalTase mRNA in B cells from patients with RA and normal controls
The level of PBMC (total cells isolated using lymphoprep) β-1,4-GalTase mRNA was found to be reduced significantly in eight patients with RA compared with eight normal controls (P < 0.005, data not shown). However, β-1,4-GalTase mRNA levels were not reduced significantly in either peripheral blood lymphocytes (non-adherent PBMC) from six patients with RA compared with six normal individuals (P > 0.9, data not shown), or in B cells (CD19+ cells) from 14 patients with RA compared with 13 normal controls (P > 0.7, Fig. 1a).

IgG galactosylation levels in patients with RA
The percentage of oligosaccharide chains lacking galactose was significantly higher in the sera from the patients with RA than in the normal subjects in all the studies conducted (Fig. 1b). No relationship was found between the level of IgG galactosylation and the level of CD19+ B-cell β-1,4-GalTase mRNA (r = 0.27, P > 0.2).

β-1,4-GalTase gene expression in B cells from MRL-lop mice
The arthritis-prone MRL-lop strain mice, the congenic strain...
and IgG-negative non-adherent cells (Ig G- ) from spleen and lymph nodes of MRL-1pr (solid bars), MRL-1+/+ (hatched bars), and CBA/Ca strain mice (arbitrarily assigned a value of 100 for each experiment). Error bars indicate ± standard error of the mean for three separate RPA measurements on each sample. Each sample contains total RNA from pools of seven mice.

MRL-1+/+ and the CBA/Ca strain with normally galactosylated IgG, all showed similar levels of β-1,4-GalTase mRNA in surface-IgG-positive splenic B cells. However, β-1,4-GalTase mRNA levels were reduced in surface-IgG negative non-adherent spleen cells from both MRL-1pr and MRL-1+/+ strain mice compared with CBA/Ca mice (Fig. 2). In the lymph node compartment, β-1,4-GalTase mRNA levels were again comparable in surface-IgG positive cells from all three strains of mice. For the surface-IgG negative non-adherent lymph node cells, the mRNA levels for this enzyme were much reduced in MRL-1pr mice compared with that in both MRL-1+/+ and CBA/Ca strain mice (Fig. 2).

Distribution of G0-secreting B cells in arthritic versus control mice

In MRL-1pr mice the G0 values of the secreted IgG in peripheral blood and spleen cell cultures were significantly higher when compared with bone marrow (P = 0.007 and P = 0.004 respectively, Fig. 3) but not lymph node (P > 0.2). We also measured the concentration of total IgG secreted spontaneously in cell cultures from four different lymphoid compartments. The only significant difference was the increased level of secretion by the spleen suspensions compared with the bone marrow (P = 0.024, data not shown).

In the control strain CBA/Ca, the amount of IgG secreted and the percentage of IgG G0 were not significantly different in any of the compartments studied.

When comparing the two strains, G0 levels of secreted IgG from peripheral blood and spleen cell cultures (but not from bone marrow or lymph node) were significantly higher in MRL-1pr mice (P = 0.041 and P = 0.006 respectively) reflecting the increased serum IgG G0 levels in these mice (P < 0.001). The production of IgG (IgG concentration) was raised significantly only in the MRL-1pr splenic cell supernatants when compared with CBA/Ca (P = 0.03).

**DISCUSSION**

In this study, we show that peripheral blood and spleen cell cultures from MRL-1pr mice secrete galactosyl IgG in vitro. We have also shown previously that immunoglobulins newly secreted from PWM-stimulated lymphocytes from RA patients have reduced levels of galactose. These data imply that the defect in IgG galactosylation is at least in part attributable to the biosynthetic pathways. Galactose is added to the oligosaccharide chains of IgG by the enzyme β-1,4-GalTase in trans-Golgi in the Golgi complex. Not much is known about the control of glycosylation. Although there is extreme microheterogeneity in the structure of the oligosaccharides of IgG, this variation is not completely random. For example, it was shown that the molar ratio of galactosylated, monogalactosylated, and digalactosylated oligosaccharides of IgG is relatively constant in normal healthy individuals of similar age. Therefore, potent control mechanisms must regulate the oligosaccharide synthesis machinery. Part of this control comes from the specificity and the amount of glycosyltransferases present (cell- and tissue-specific glycosylation), the primary peptide structure, and the constraints imposed by the three-dimensional structure of individual proteins.

The defect in galactosylation in RA seems to be restricted to IgG. Transferrin, IgA, and the Fab of IgG30,31 have almost fully galactosylated oligosaccharides in patients with RA. Galactosylation at the Fc site of IgG is complicated by the unique structure of IgG in that unlike other glycoproteins that have their carbohydrates exposed, the IgG oligosaccharide moiety is contained within the space between the Fc C2 polypeptide domains. Therefore, it could be that galactosylation at this site is more sensitive to small fluctuations in the level of enzyme β-1,4-GalTase, or alternatively the enzyme level is not the limiting factor but the rate of H−H−chain disulfide bond formation in the Golgi determines the efficiency of galactosylation at this site. Although glycosyltransferases, in general, are thought to be regulated at the transcriptional level, there is some evidence for post-translational regulation of the β-1,4-GalTase enzyme. This enzyme has been shown to be phosphorylated at serine residues and there is some evidence that a cdc-related protein kinase, p58 galactosyltransferase-associated protein kinase, may be involved in the regulation of β-1,4-GalTase activity through phosphorylation. The suggestion that β-1,4-GalTase enzyme activity is
reduced in RA is controversial. Data on β-1,4-GalTase enzyme activity^{8,10,35,36} suggest that the glycoprotein acceptor and/or the method of B-cell preparation used in each study contribute to the observed activity of the β-1,4-GalTase enzyme. Our observation of similar levels of β-1,4-GalTase mRNA in peripheral CD19^+ B cells from patients with RA and normal controls, and also in both splenic and lymph node sIgG^+ B cells from arthritis-prone and control mice suggest that the B-cell β-1,4-GalTase activity, if reduced in RA, must be post-transcriptionally regulated.

We cannot exclude the possibility that mRNA levels for the enzyme in circulating peripheral blood B cells, the majority of which are resting virgin and memory cells, might not reflect the levels of the message in the plasma cells which are the source of the defectively glycosylated serum IgG. It is not possible to directly isolate sufficient numbers of plasma cells to provide enough RNA for the RPA measurements, and the use of in vitro techniques such as Epstein–Barr virus (EBV) transformation has been shown to increase β-1,4-GalTase activity in RA B cells leading to the conclusion that such procedures can not be used in these types of study.\(^\text{37}\) An alternative approach might be to use reverse transcriptase-polymerase chain reaction (RT-PCR) but such assays are accurately described as 'semi-quantitative' and are unlikely to provide as accurate a quantitation of mRNA levels as the RPA. All previous studies on β-1,4-GalTase activity in RA have examined B lymphocytes rather than plasma cells and the aim of the present study was to establish if the reported decrease in β-1,4-GalTase activity in this cell type in RA can be explained by a decreased production (or alternatively increased turn-over) of the specific mRNA species. We clearly show this not to be the case.

Our finding of comparable levels of β-1,4-GalTase mRNA in IgG-positive B cells from spleen and lymph nodes of MRL-lpr mice support the observations of Axford and co-workers\(^\text{20}\) who have reported a reduction in peripheral but not splenic B-lymphocyte β-1,4-GalTase activity in MRL-lpr mice compared with CBA/Ca mice, and no significant difference in B-cell GalTase activity when comparing MRL-lpr mice with MRL/+/+.

Previously we have reported that there are reduced levels of β-1,4-GalTase mRNA in splenic lymphocytes (spleen cell suspension depleted of plastic-adsorbed cells) from MRL-lpr mice.\(^\text{21}\) This has been confirmed and reflected here in decreased levels of the message in the IgG-negative population (cell suspension depleted of plastic-adherent cells and sIgG^+ cells) from spleen and lymph nodes, suggesting a further abnormality in MRL-lpr T cells. Our findings would correspond with those of Imai and colleagues\(^\text{27}\) who report reduced binding to lpr lymph node T cells of the lectins RCA and allo A, which bind primarily to a Galβ1-4GlcNAc structure. Contrary to this observation, the same authors reported an increase in the activity of β-1,4-GalTase in MRL-lpr lymph node T cells using asialo-agalacto-transferrin as the acceptor.\(^\text{33}\) Whether this and other glycosylation abnormalities in MRL-lpr T cells\(^\text{38-40}\) are associated with the defect in fas-mediated apoptosis in B220^+, CD4^−, CD8^− (DN) T cells\(^\text{41}\) remains to be established.

We have found no association between β-1,4-GalTase mRNA levels or enzyme-activity levels\(^\text{32}\) and IgG glycosylation in RA B cells. Recent evidence obtained using lymphoblastoid cell lines also points to a lack of correlation between β-1,4-GalTase enzyme-activity levels and the extent of galactosylation.\(^\text{42}\) It may be that β-1,4-GalTase enzyme levels are not directly related to the galactosylation defect of IgG, and that some other abnormality in IgG biosynthesis or regulation of glycosylation may be causing the defective IgG galactosylation seen in RA.

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