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Glycosylation of Immunoglobulin G in Cerebrospinal Fluid and Multiple Sclerosis

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Abstract

The glycosylation features of CSF oligoclonal IgG, and possible changes in *N*-glycans of CSF IgG in multiple sclerosis (MS) were studied.

After isoelectric focusing (IEF) of CSF, bands were detected using biotinylated lectins and avidin-horseradish peroxidase. Concanavalin A (Con A) binding showed that mannose exists throughout the pH range of oligoclonal IgG. Sambucus nigra antigen (SNA) bound acidic and neutral oligoclonal IgG only, suggesting that alkaline oligoclonal IgG is deficient in sialic acid. Deglycosylation of CSF IgG using peptide-*N*-glycosidase F suggested that the range of isoelectric points of oligoclonal IgG bands is not due to carbohydrate differences alone.

Lectin immunoassays, whereby protein A purified IgG was captured by anti-IgG coated tubes and probed using a range of biotinylated lectins, were used to compare 13 CSF samples from MS patients with 14 control samples. With Con A binding, a significantly higher mean and larger variance was found for the MS group (t-test: $P<0.05$). Con A binding correlated with CSF [IgG]/[total protein]% ($r=0.390$; $P=0.0443$).

Using HPLC to separate oligosaccharides released from IgG by hydrazinolysis and labelled with 2-aminobenzamide, glycans were determined in 7 CSF samples with oligoclonal IgG, and 6 CSF samples without. The ratio of the peak for biantennary fucosylated agalactosyl glycans to total monogalactosylated glycan peaks was lower for the oligoclonal IgG samples (t-test: $P=0.0141$).

The overall results suggested that glycosylation changes occur in CSF IgG in MS, and that oligoclonal IgG contains less sialic acid but more galactose than polyclonal IgG.

Contents

Abbreviations

2AB	2-aminobenzamide
3-D	three dimensional
ABEI	aminobutylethyl isoluminol
ADP	adenosine diphosphate
ADP	adenosine diphosphate
AEC	anion-exchange chromatography
AFP	alpha-fetoprotein
al	alia
AMP	adenosine monophosphate
ANOVA	analysis of variance
AP	alkaline phosphatase
Ara	arabinose
Asn	asparagine
ATP	adenosine triphosphate
BSA	bovine serum albumin
BSL II	*Bandeiraea simplicifolia* Lectin
C	constant (of domain in immunoglobulins)
CA	carcino antigen
cAMP	cyclic adenosine monophosphate
CD	calcium dependent
CE	capillary electrophoresis
CEA	carcino-embrionic antigen
CIMS	chemical ionisation mass spectrometry
CMP	citidine monophosphate
CNS	central nervous system
Con A	concanavalin A
CRP	C-reactive protein
CSF	cerebrospinal fluid
CSL	cerebellar soluble lectin
CT	computerised tomography
CV	coefficient of variation
CZE	capillary zone electrophoresis
Da	Dalton
DAB	3,3'-diaminobenzidine-tetrahydrochloric acid
DBA	*Dolichos biflorus* agglutinin
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
Dol-P-P	dolichol pyrophosphate
DP	degradation products
DSL/DSA	*Datura stramonium* lectin/agglutinin
EAE	experimental allergic encephalomyelitis
EBNA-1	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
ECL	*Erythrina cristagalli* lectin
EDSS	expanded disability status scale
EGS	ethylene glycol-succinic acid ester
EIMS	electron impact mass spectrometry
ELISA	enzyme-linked immunosorbent assay
EPO	erythropoietin

ER	endoplasmic reticulum
ES	electrospray ionisation
FAB	fast atom bombardment
Fab	variable fragment (of immunoglobulin)
FACE	fluorophore-assisted carbohydrate electrophoresis
Fc	constant fragment (of immunoglobulin)
FSH	follicle-stimulating hormone
Fuc	fucose
Gal 0	agalactosyl
Gal 1	monogalactosyl
Gal 2	digalactosyl
Gal	galactose
GalC	galactocerebroside
GalNAc	*N*-acetylgalactosamine
GC	gas chromatography
GDGS	carbohydrate-deficient glycoprotein syndrome
GH	growth hormone
Glc	glucose
GlcNAc	*N*-acetylglucosamine
GLIA	glycoprotein-lectin immunosorbent assay
GPC	gel permeation chromatography
GPI	glycosylphosphatidylinositol
GSL	*Griffonia simplicifolia* lectin (BSL)
GU	glucose units
H	heavy (immunoglobulin chain)
h	hour
HCG	human chorionic gonadotropin
HEMPAS	hereditary erythroblastic anaemia with multinuclearity and a positive serum lysis test
HIV	human immunodeficiency virus
HLA	leucocyte histocompatibility antigens
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HSA	human serum albumin
Hyl	hydroxylysine
Hyp	hydroxyproline
ICAM	immunoglobulin-like cell adhesion molecule
IEC	ion-exchange chromatography
IEF	isoelectric focusing
IFN	interferon
IgA	immunoglobulin A
IgD	immunoglobulin D
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IT	immunoturbidimetry
kDa	kiloDalton
L	light (immunoglobulin chain)
LacNAc	*N*-acetyllactosamine
LCA	*Lens culinaris* agglutinin
Le	Lewis antigen

LEL	*Lycopersicon esculentum* lectin
LH	luteinizing hormone
LIA	luminescence immunoassay
MAG	myelin-associated glycoprotein
MALDI	matrix assisted laser desorption ionisation
Man	mannose
MBP	myelin basic protein
MHz	megahertz
min	minute
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
MS	mass spectrometry
MS	multiple sclerosis
NADH	reduced nicotinamide adenine dinucleotide
NAL	*N*-acetyllactosamine
NB-DNj	*N*-butyldeoxynojirimycin
N-CAM	neural cell adhesion molecule
NeuAc	*N*-acetylneuraminic acid
N-linked, *N*-Glycans	linkage through amide nitrogen of asparagine
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
OID	other inflammatory diseases
O-linked, O-Glycans	linkage through hydroxyl group of an amino acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS-G	phosphate buffered saline with gelatine
PBS-T	phosphate buffered saline with Tween 20
PHA	*Phaseolus vulgaris* agglutinin
pI	isoelectric point
PLP	proteolipid protein
PNA	peanut agglutinin
PNGase F	peptide-*N*-glycosidase (*Flavobacterium*)
PNS	peripheral nervous system
PP	primary-progressive
PR	progressive-relapsing
PSA	*Pisum sativum* agglutinin
PSA	polysialic acid
PUFA	polyunsaturated fatty acids
PVDF	polyvinylidene fluoride
r	correlation coefficient
RA	rheumatoid arthritis
RAAM	reagent analysis array method
RCA	*Ricinus communis* agglutinin
REGA	remnant epitopes generate autoimmunity
RF	rheumatoid factor
RIA	radioimmunoassay
RID	radial immunodiffusion
RLU	relative luminescence units
RNA	ribose nucleic acid
RR	relapsing-remitting
RT	room temperature

s	second
SBA	soybean agglutinin
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
Ser	serine
SiaLe	sialyl Lewis antigen
SNA	*Sambucus nigra* agglutinin
SP	secondary-progressive
SpA	protein A
STL	*Solanum tuberosum* lectin
TBS	tris buffered saline
TFA	trifluoroacetic acid
TFMS	trifluoromethanesulphonic acid
Thr	threonine
TOF	time of flight
TSH	thyroid stimulating hormone
Tyr	tyrosine
UDP	uridine diphosphate
UEA	*Ulex europaeus* agglutinin
V	variable (of domain in immunoglobulins)
V	Volt
VVL	*Vicia villosa* lectin
WGA	wheat germ agglutinin
Xaa	any amino acid except proline
Xyl	xylose

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Chapter One

Introduction

1.1. The Dawning of Glycobiology

In the last thirty years there has been soaring interest in the relatively small, but highly variable, carbohydrate components of glycoproteins and other glycoconjugates. These oligosaccharide chains are fundamental to the function and structure of proteins and variations modify a protein's behavior. Moreover, changes in oligosaccharides are associated with disease states, and from this has arisen the concepts that manipulation and modification of sugar structures might result in therapeutic benefits, or that they might make useful diagnostic tools (Dwek, 1995a).

1.1.1. Early History

The recent development of sensitive methods to elucidate the chain structures has opened up the road of glycobiology research. Previously, during the long history of protein biochemistry, the functional aspect of the sugar moieties was largely ignored, mainly because of the difficulty in assaying the small sugar chains associated with the large protein structures (Kobata, 1998). For many years, carbohydrates were ill-defined components of glycoproteins, and were even thought to be contaminants. The agglutination properties of lectins had been known for many years (Stillmark, 1888; Sumner and Howell, 1936) as was their ability to distinguish different blood groups (Boyd and Sharpleigh, 1954). By the early nineteenth century, carbohydrates were being isolated from mucins and other proteins and in the 1930s mannose, galactose and glucosamine were characterized (Gottschalk, 1972). One of the first glycoproteins to be investigated was ovalbumin, from hen's egg white. It was shown in 1965 to have a carbohydrate

moiety which was attached at a given position of the polypeptide chain, and which was often variable in structure from one ovalbumin molecule to another (Cunningham et al, 1965). Thus the concept of 'microheterogeneity' was born.

1.1.2. Glycoproteins and Disease

It was realized that carbohydrate composition could vary with different diseases. Assays measuring serum sialic acid (Svennerholm, 1958) were developed, which was found to be increased in cancer, and sialic acid-deficient α_1-acid glycoprotein was shown to be produced in certain pathological states (Schmid, 1964). Wheat germ agglutinin was found to agglutinate malignant cells, but not normal ones (Aub, 1965) and histological stains based on lectins were developed which could distinguish pathological tissue from normal (Riley and Elhay, 1996).

Various chemical methods were developed to analyze glycosylation, such as hydrazinolysis to release N-linked chains from glycoproteins (Yosizawa et al, 1966) and periodate oxidation to sequence the oligosaccharides determined (Neuberger and Marshall, 1972). By the 1970s the structures of many of the carbohydrate groups of glycoproteins had been determined, though their function was somewhat a mystery. Nevertheless, numerous papers were published describing variations in carbohydrate composition of glycoproteins related to specific diseases, such as IgG in rheumatoid arthritis (Mullinax, 1975), fucosylated alpha-fetoprotein in liver disease (Aoyagi, 1985), and acute-phase proteins in cancer (Turner et al, 1985). Abnormal heterogeneity of serum transferrin was found in alcoholics (Stibler, 1980). Microheterogeneity was also demonstrated in glycoprotein hormones (Kerckaert et al, 1979) using lectin affinity chromatography or lectin affino-electrophoresis (Bøg-Hansen, 1973). Lectins were still very important tools in the characterization of glycoproteins (Sharon and Lis, 1972; Goldstein and Poretz, 1986). As analysis became more and more sophisticated, and new techniques, such as X-ray crystallography, high pressure liquid chromatography (HPLC),

mass spectrometry (GC-MS), and nuclear magnetic resonance (NMR) came to be developed, different glycoproteins and different diseases were looked at. But none has been as extensively researched as IgG in rheumatoid arthritis, which is described below.

1.1.3. Galactose-Deficient IgG

For some years it had been known that the serum of rheumatoid arthritis (RA) patients contained rheumatoid factor (RF), consisting of IgM and IgG molecules which bound to human IgG. It was known that the reaction with IgG took place only if the IgGs were immobilized by adsorption to red blood cells (Waaler, 1940; Rose et al, 1948), to latex particles (Singer et al, 1962), or if the IgGs themselves were aggregated by heat (Normansell, 1971). The adsorption or heating of IgG was necessary, causing a change in the configuration of the IgG molecule to reveal reactive determinants, present in a cryptic state on native IgG. These determinants were identified as carbohydrate residues on the Fc fragment (Clamp and Putnam, 1964; Hunneyball and Stanworth, 1976). Later it was realized that carbohydrate was necessary for the formation of an IgG-IgG complex (Hymes et al., 1978) and that carbohydrate residues influence the secondary structure of IgG (Duc Dodon and Quash, 1981).

In 1975 it was reported that there was a decrease in the galactose content of IgG in patients with rheumatoid arthritis (Mullinax, 1975). In 1985, scientists collaborating in Oxford, Birmingham and Tokyo presented a fundamental paper in Nature based on the analysis of the primary sequences of about 1400 oligosaccharides chains from 46 IgG samples (Parekh et al, 1985). They used various techniques: controlled hydrazinolysis to release the intact chains; reduction of the terminal *N*-acetylglucosamine (GlcNAc) residues using NaB^3H_4 to radioactively label each chain; neuraminidase digestion to analyze the distribution of neutral structures, then fractionation of the resulting 'asialo' oligosaccharide mixtures by gel filtration chromatography. Later it was shown that there was reduced galactosyltransferase activity in the Golgi bodies of B-cells in rheumatoid

arthritis giving rise to the galactose deficient glycans of IgG (Axford et al, 1987). It was also found that the observed remission of the disease in pregnancy corresponded to increased activity of this enzyme (Pekelharing et al, 1988). Agalactosyl IgG was associated with juvenile onset, as well as adult, rheumatoid arthritis, and its prevalence correlated with disease activity (Parekh et al, 1988). Further work on IgG showed that the galactosylation of IgG N-linked oligosaccharides decreased as a parabolic function of age, and a parallel was seen with the decrease of immunological competence with age (Parekh et al, 1988).

1.1.4. Further Glycoprotein Studies.

Abnormal lectin binding to IgG was demonstrated in the sera (Malaise, 1987; Parkkinen, 1989; Sumar, 1990) and synovial fluid (Casburn-Budd, 1990) of rheumatoid arthritis patients. IgG and other proteins were shown to be abnormally glycosylated in other inflammatory diseases such as osteoarthritis (Carter et al, 1989), systemic lupus erythematosus and Crohn's disease (Tomana et al, 1988), Sjögren's syndrome, sclerodermic disorders and mixed connective tissue diseases (Silvestrini et al, 1989). The glycosylation of IgA has been studied in relation to various diseases, notably nephropathy (Baharaki et al, 1996). Various acute-phase proteins have been investigated: for example α_1-acid glycoprotein glycoforms in relation to inflammation (De Graaf et al, 1993; Lacki et al, 1994; van Dijk et al, 1994), cancer (Mackiewicz and Mackiewicz, 1995) and tuberculosis (Fassbander et al, 1995); haptoglobin with respect to cancer (Katnik et al, 1992) and rheumatoid arthritis (Thompson et al, 1993), and α_1-antichymotrypsin with liver diseases (Hachulla et al, 1992). Transferrin has been particularly studied in relation to liver disease (Matsumoto et al, 1994), alcohol abuse (Stibler et al, 1980) and carbohydrate-deficient glycoprotein syndromes (Stibler and Jaeken, 1990). The different glycoforms of alkaline phosphatase have provided a means of distinguishing between liver and bone isoforms (Chamberlain et al, 1992; Crofton, 1992). Glycosylation changes in various

proteins in cancer have been extensively investigated, notably alpha-feto protein (Marrink et al, 1990; Aoyagi et al, 1990) and chorionic gonadotropin (Kobata, 1998). Other hormones such as thyroid stimulating hormone (TSH) (Papandreou et al, 1993), luteinizing hormone (LH), follicle-stimulating hormone (FSH) and erythropoietin (EPO) (Storring, 1992) have been shown to differ in their activities *in vivo* and *in vitro*, according to their carbohydrate moieties.

1.1.5. Glycobiology.

The glycosylation aspects of some diseases will be looked at in more detail in Section 3. The above examples are a small part of the whole picture of glycoconjugates and glycosystems that has been uncovered in the last thirty years. The ability to accurately sequence the oligosaccharide units of glycoconjugates has revealed the remarkable complexity and diversity of these molecules which can affect every aspect of biological function (Varki, 1993).

In addition to glycoproteins there are a number of other types of glycoconjugates whose oligosaccharides are not always the discrete, specific and conserved structures which are found in proteins and glycopeptides (Kobata, 1994). Many lipids, such as the phosphoglycerides and the glycosphingolipids, contain carbohydrates. These have mainly a structural or organizational function in the cell, such as the glycosylphosphatidylinositol (GPI) membrane anchors which are interposed through the lipid bilayer affording a stable association of the membrane and a protein (Ferguson, 1991). Another large group of glycoconjugates is the proteoglycans, containing a larger fraction of carbohydrate than glycoproteins in the form of glycosaminoglycan chains covalently linked to a protein core. These have roles of lubricants and support elements in the body, but also exist as heparin and other anti-coagulation agents (Yanagishita and Hascall, 1992). Lectins exist in plants, animals and bacteria, as defence mechanisms, as mediators in cell to cell interactions or as receptors within or outside cells. For instance, the selectins mediate interactions between

circulating leukocytes and endothelial cells at sites of inflammation and in lymph nodes, causing the leukocyte to roll along the surface of the epithelium (Lasky, 1992; Springer, 1991). Galectins bind β-galactosides within the cell, possibly regulating RNA transcription, and at the cell surface are involved in cellular differentiation during growth (Barondes et al, 1994). Collectins and mannose receptors in animals recognize exogenous sugars present on potential microbial pathogens (Drickamer and Taylor, 1993; Weis and Drickamer, 1994), and carbohydrate-binding properties have been detected in cytokines such as interleukin 1 and 2 (Brody and Durum, 1989). With respect to neurology, mannose-binding cerebellar soluble lectin (CSL) on cell surfaces is important both for cell adhesion and signaling between cells (Zanetta et al. 1987). Antibodies to CSL cause demyelination and have been implicated in multiple sclerosis (Zanetta et al. 1990). Neural cell adhesion molecule (N-CAM) which belongs to the immunoglobulin-superfamily, carries long chains of polysialic acid (PSA) which allow binding between cells. (Seki and Arai, 1993) Other members of this family, which actually bind sialic acids, are sialoadhesin, CD22, CD33 and myelin associated glycoprotein (MAG) (Powell and Varki, 1995).

1.1.6. This Project.

It can be seen that the field of glycobiology extends across a range of disciplines in biological sciences, and a review of the whole area is beyond the scope of this thesis. Attention will be focused on glycosylation with relevance to diseases and diagnosis and, more specifically, to the *N*-glycosylation of human IgG and changes that may be found in multiple sclerosis.

1.2. Structures and Functions of the Sugar Chains.

There follows a brief description of the sugar chains of glycoproteins. The aspects which relate most to this project, i.e. the glycosylation of IgG, will be covered in

greatest depth. N-linked glycans will be described in more detail than O-linked, even though O-linked are just as widespread and biologically important.

1.2.1. Prevalence.

Glycoproteins vary considerably in their carbohydrate content: less than 1% in some of the collagens, more than 99% in glycogen. They also differ in the number of their monosaccharide units, which ranges from one up to two hundred for most glycoproteins, although some, such as glycogen, contain many thousands. These monosaccharides are usually in the form of oligosaccharide or polysaccharide chains, linear or branched, and often referred to as glycans. Branches are very seldom crosslinked (Sharon and Lis, 1997).

A feature of glycans is their heterogeneity. Individual molecules of a given glycoprotein may carry different saccharides at the same attachment site of the polypeptide chain. This contrasts to the uniformity of the polypeptide chains to which they are attached. For example, IgG contains an average of 3 glycans per molecule, with more than 30 different glycan structures possible (Dwek, 1995). However the ratio of the different structures for the sum of all molecules in a sample is relatively constant. Moreover, a similar heterogeneity is found upon analysis of monoclonal IgG, showing that the large number of different structures found with IgG is not due to polyclonicity. Heterogeneity results from major or minor changes in glycan structure, such as increased branching, loss of one or more arms, or deletion or addition of one or more carbohydrate units, producing discrete molecular subsets of a glycoprotein, often called glycoforms. In IgG the number of glycoforms could be 900. Different forms of glycan chains produce different effects on a protein, altering strengths or positions of charges and so causing a different confirmation of the tertiary structure of the protein. So various glycoforms of a protein may have different physical and biochemical properties, which might produce a functional diversity.

1.2.2. Carbohydrate Complexity

Carbohydrates, through their unique multi-linkages of both monomer and branching structures, contain potentially more information, in a short sequence, than any other biological oligomer (Laine 1994). Other biopolymers such as nucleic acids and proteins are composed of covalently linked units. For example, a pair of amino acids will combine to make two structures, at most. But, taking a pair of monosaccharides such as *N*-acetylglucosamine (GlcNAc) and mannose: GlcNAc can bind to any one of 4 hydroxyl groups on mannose; two anomeric linkages exist for the GlcNAc link, making 8 possibilities; furthermore, the GlcNAc might exist in the furanose or pyranose form, making 16 possibilities. Table 1.1 shows the number of combinations of units found in similar lengths of DNA, proteins and oligosaccharides.

Table 1.1. Number of linear oligomers of length N (Kobata, 1994)

N	DNA	Proteins	Oligosaccharides	
			O=4*	O=8*
1	4	20	4	8
2	16	400	128	800
3	64	8000	4096	6.40×10^4
6	4096	6.40×10^7	1.34×10^8	3.27×10^{10}
10	1.04×10^6	1.28×10^{13}	1.40×10^{14}	1.34×10^{18}

* =*Number of monosaccharide types*

Another important distinction between these biopolymers is that, while nucleic acids and proteins are direct gene products i.e. arise from a DNA template, oligosaccharide sequence is determined at the time of synthesis and allows for considerable variation, with possible influence from the environment.

1.2.3. Monosaccharides

The commonly occurring monosaccharides found in glycoproteins are shown in Table 1.2. Of these, galactose, mannose and *N*-acetylglucosamine are very common in most organisms. Glucose is largely confined to a small number of animal glycoproteins (mainly collagens).

Table 1.2. Common monosaccharide constituents of glycoproteins (Sharon and Lis, 1997).

Class	Compound[a]	Abbreviation	Comments
Hexoses	D-Galactose	Gal	
	D-Glucose	Glc	Mainly in collagens
	D-Mannose	Man	
Deoxyhexoses	L-Fucose	Fuc	
Pentoses	L-Arabinose	Ara	In plant glycoproteins
	D-Xylose	Xyl	In proteoglycans and plant glycoproteins
Hexosamines	N-Acetyl-D-galactosamine	GalNAc	
	N-Acetyl-D-glucosamine	GlcNAc	
Uronic acids	D-Glucuronic acid	GlcA	In proteoglycans
	L-Iduronic acid	IdoA	In proteoglycans
Sialic acids	N-Acetylneuraminic acid[b]	Neu5Ac	In vertebrates and higher invertebrates

[a] *The ring form is usually pyranose, although furanose forms do occur.*
[b] *Other sialic acids are also found in glycoproteins.*

N-acetylgalactosamine and uronic acids exist mainly in animals, rarely in plants. Fucose is common to plants and animals. Xylose is in plant glycoproteins, but animal proteoglycans. Arabinose is confined to plants. Sialic acids are found in most abundance in higher invertebrates and vertebrates. While *N*-acetylneuraminic acid occurs widely, other sialic acids have a limited distribution. Figure 1.1 shows the structures of the main monosaccharides in eukaryotes. D-glucose most frequently serves as the starter molecule for the biosynthesis of all monosaccharides and amino sugars.

1.2.4. Carbohydrate-peptide linkage

There are three main types of linkage between glycans and polypeptides. It is only the *N*-glycosidic type which has been found to date in human IgG, and will be dealt with here in more depth than other linkages. The more common *N* and *O* types are summarized in Table 1.3.

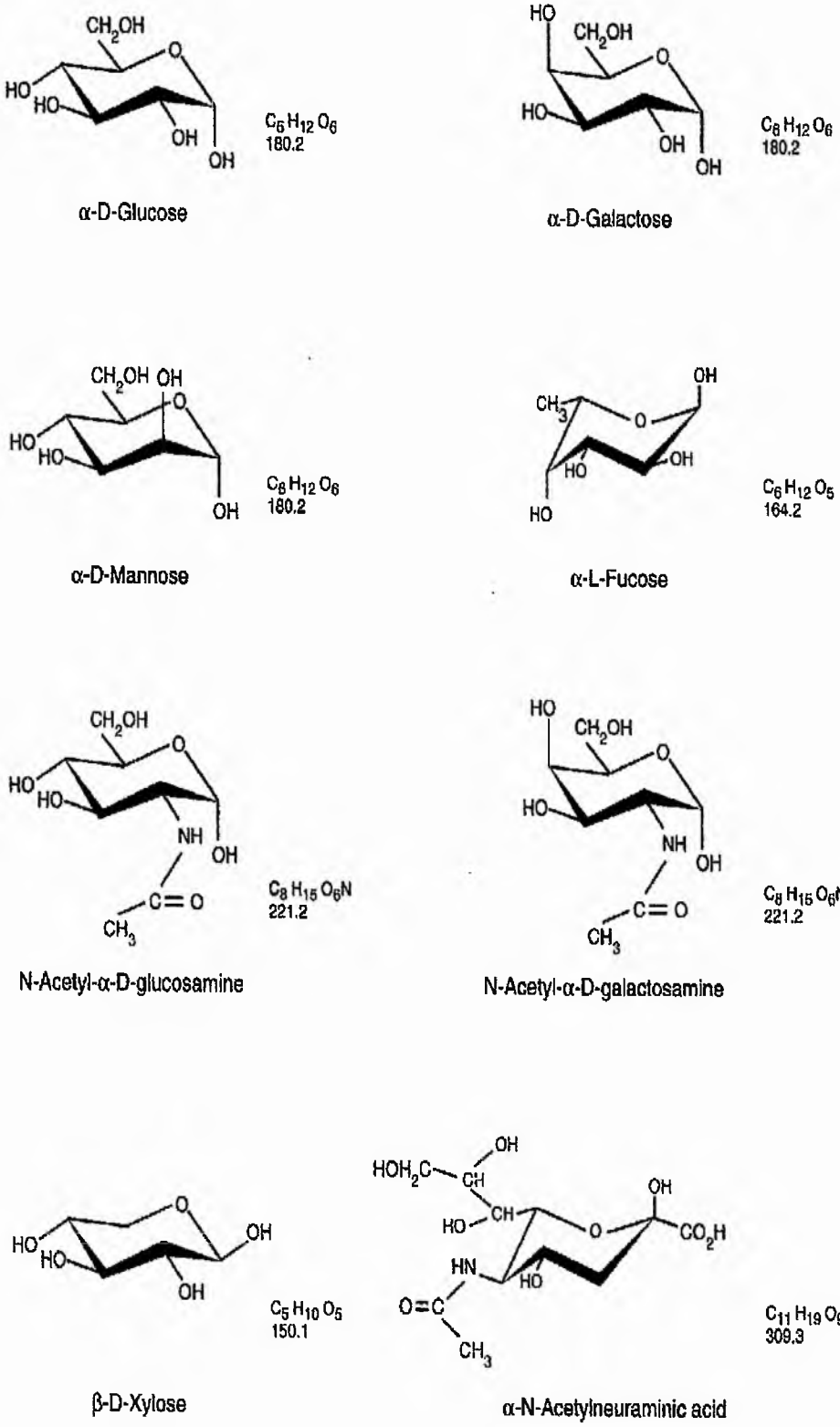


Figure 1.1. Structures of monosaccharides commonly found in eukaryotic glycoproteins (Oxford GlycoSystems, 1994a).

Table 1.3. Carbohydrate-peptide linking groups (Sharon and Lis, 1997).

Type	Monosaccharide	Amino acid	Occurrence
N-glycosidic	β-GalNAc	Asn	Archaebacteria
	β-Glc	Asn	Animals[a]
	β-Glc	Asn	Archaebacteria
	β-GlcNAc	Asn	Common
	Rhamnose	Asn	Eubacteria
O-glycosidic	Ara	Hyp	Plants
	α-Fuc	Ser, Thr	Animals
	α-Gal	Hyp	Plants, eubacteria
	α-Gal	Ser	Plants, eubacteria
	β-Gal	Hyl	Animals[b]
	β-Gal	Tyr	Eubacteria
	α-GalNAc	Ser, Thr	Common
	α-Glc	Tyr	Animals[c]
	β-Glc	Ser, Thr	Eubacteria, animals
	β-GlcNAc	Ser, Thr	Animals
	α-Man	Ser, Thr	Yeasts, animals
	β-Xyl	Ser	Animals[d]

[a] To date found only in laminin
[b] Found only in collagens
[c] Only in glycogen
[d] Confined to proteoglycans

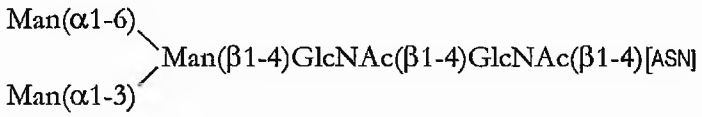
1. *N*-Glycosidic. This occurs between the reducing terminal sugar and the amide group of asparagine, producing *N*-glycans. The most common linking group is *N*-acetyl-glucosamine-asparagine (GlcNAc-Asn). The asparagine is part of the consensus sequence (sequon) Asn-X-Ser/Thr, where X may be any amino acid except proline. Not all such residues however are glycosylated. During the last decade new carbohydrate-asparagine linking groups have been discovered, mainly in bacterial glycoproteins, where GlcNAc is replaced by other monosaccharides, as shown in Table 1.3.
2. *O*-Glycosidic. This will occur between the terminal sugar and a hydroxyl group of an amino acid, most commonly serine or threonine. A large variety of *O*-glycosidic linkages exist (Table 1.3), including the well-known GalNAc-Ser/Thr linking group found in numerous animal glycoproteins.

3. Other. A linkage in which α-mannose is attached *C*-glycosidically to the indole ring of a tryptophan has been discovered in human RNase U_s (Hofsteenge et al, 1994). Also, a covalent linkage of the 3-hydroxyl of ribose with glutamic (or aspartic) acid, arginine or cysteine is involved in ADP-ribosylation (Burnette, 1994).

1.2.5. Oligosaccharide Linkages

1.2.5.1. N-Linked

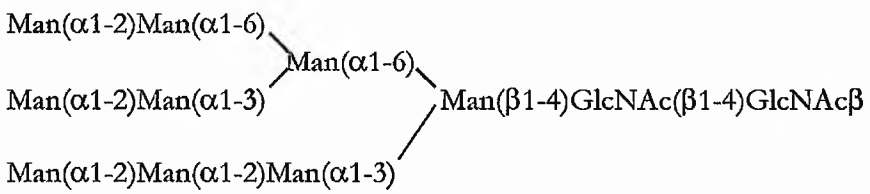
The asparagine-linked oligosaccharides, notwithstanding all their heterogeneity, have a common core:



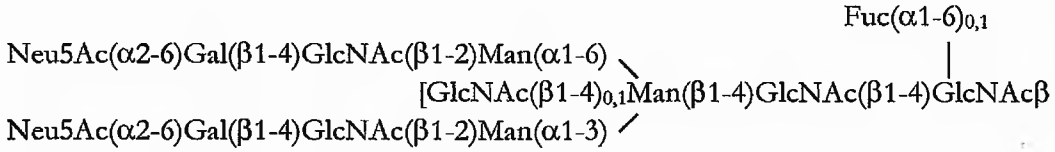
This structure is often referred to as the 'pentasaccharide core' (Sharon and Lis, 1997) or the 'trimannosyl core' (Brockhausen, 1993). *N*-linked glycans are usually classified into three groups: oligomannose, complex and hybrid, to which a fourth, xylose containing, has recently been added (Vliegenthart and Montreuil, 1995) (Table 1.4). Oligomannose glycans usually contain 2 to 6 α-mannose residues in these branches, although some yeasts produce chains with up to 200 mannose units. Complex glycans contain up to five units of the disaccharide Gal(β1-4)GlcNAc (sometimes referred to as *N*-acetyllactosamine, LacNAc or NAL in brief), β-linked to the two α-mannoses of the trimannosyl core. These often have terminal sialic acids. Further variations occur with possible additions of fucose to the core or to outer branches, of a 'bisecting' GlcNAc linked β1-4 to the β-mannose of the trimannosyl core, or of Galα(1-3) to the galactose of *N*-acetyllactosamine. Hybrid glycans have features of both oligomannose and complex types. One or two α-mannose units are linked to the Manα1-6 arm of the core, with a complex chain attached to the Manα1-3 arm.

Table 1.4. Representative structures of the different types of *N*-linked glycans (Sharon and Lis, 1997)

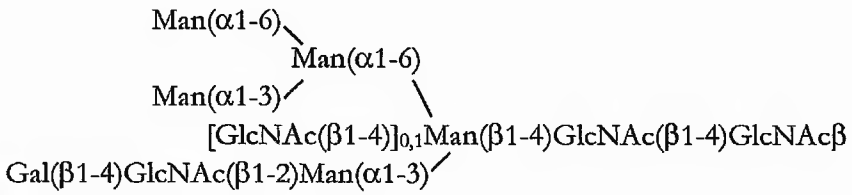
Oligomannose



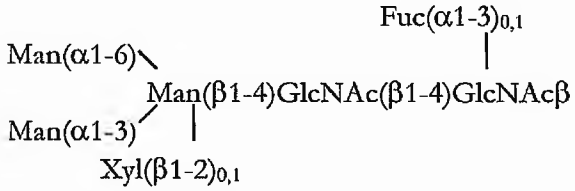
Complex



Hybrid



Xylose-containing



The subscript '0,1' indicates residue may be present or absent.

The branches of many N-glycans contain poly-N-acetyllactosamine, $[Gal\beta4GlcNAc\beta3]_n$, where n may be as high as 50. Due to the branch specificity of the enzyme that forms these - β1,3-N-acetylglucosaminyltransferase – these chains predominate on the α(1-6) linked mannose of the trimannosyl core. With substitution of galactose, a large number of different structures are formed and they serve as backbones for the ABH(O), I/i and Lewis (Le^a and Le^b) blood group determinants on human erythrocyte membrane glyco-

conjugates, as well as the Le^x and sialyl Le^x ($SiaLe^x$) determinants on granulocytes and other cells.

1.2.5.2. O-Linked

The O-linked glycans, bound to proteins via the GalNAc-Ser or GalNAc-Thr linkage, are a large heterogeneous group, divided into subgroups according to the structure of the core region, as shown in Table 1.5.

The O-Glycans vary in size from monomers to polymers, typically up to 20 monosaccharides. However, mucins, common constituents of membranes and of epithelial secretions, are very large molecules with extensive heterogeneity. Their main constituent is *N*-acetylgalactosamine, along with galactose, *N*-acetylglucosamine, fucose, various sialic acids or sulphate.

Table 1.5. Core structures of *O*-glycans (Sharon and Lis, 1997)

Core	Structure	Occurrence
1.	Gal(β1-3)GalNAcα-	Mucins and other glycoproteins
2.	GlcNAc(β1-6) | Gal(β1-3)GalNAcα-	Mucins and other glycoproteins
3.	GlcNAc(β1-3)GalNAcα-	Mucins
4.	GlcNAc(β1-6) \ GlcNAc(β1-3)GalNAcα-	Mucins
5.	GalNAc(α1-3)GalNAcα-	Glycoproteins
6	GlcNAc(β1-6)GalNAcα-	Mucins and other glycoproteins

1.2.6. Glycan Biosynthesis

Whereas nucleic acids and proteins are both linear molecules in which the component units are joined by similar bonds (phosphodiester and peptide bonds respectively), glycans are usually branched and contain many different linkages. Furthermore, whereas nucleic acids and proteins are produced from template molecules as essentially invariant copies, the components of glycans are put together sequentially, with frequent opportunities for variation. This occurs in the endoplasmic reticulum (ER) and the Golgi apparatus, where glycosidases and glycosyltransferases, firmly embedded in the endoplasmic reticulum with catalytic domains in the lumen, act on the growing oligosaccharide as it moves along the lumen of the endomembrane system (Brockhausen and Schachter, 1997). The form the glycan takes is dependent on the concentrations of these enzymes in the individual cell. This can vary with the pathological or physiological state of the cell, but may also vary between individual cells in the same state. So a range of glycan structures is produced, conferring microheterogeneity to that particular protein or proteoglycan. The character of the microheterogeneity is often species or tissue specific and can change with normal development of tissues, or with pathological changes. Possible reasons for heterogeneity will be discussed in section 1.2.9.

1.2.6.1. N-Glycan Biosynthesis

A scheme of N-glycan biosynthesis is shown in Figure 1.2. It begins in the rough ER with the co-translational transfer of a large oligosaccharide ($Glc_3Man_9GlcNAc_2$) from dolichol pyrophosphate (Dol-P-P) to an asparagine (Asn) residue in the nascent polypeptide chain (Step 1). The Asn residue must be Asn-Xaa-Ser/Thr triplet (known as a 'sequon'), where Xaa is any amino acid except proline. This is followed by removal of three glucose and four mannose residues, within the lumen of the ER and Golgi apparatus, by specific α-glucosidases (Steps 2 and 3) and α-mannosidases (Steps 4 and 5).

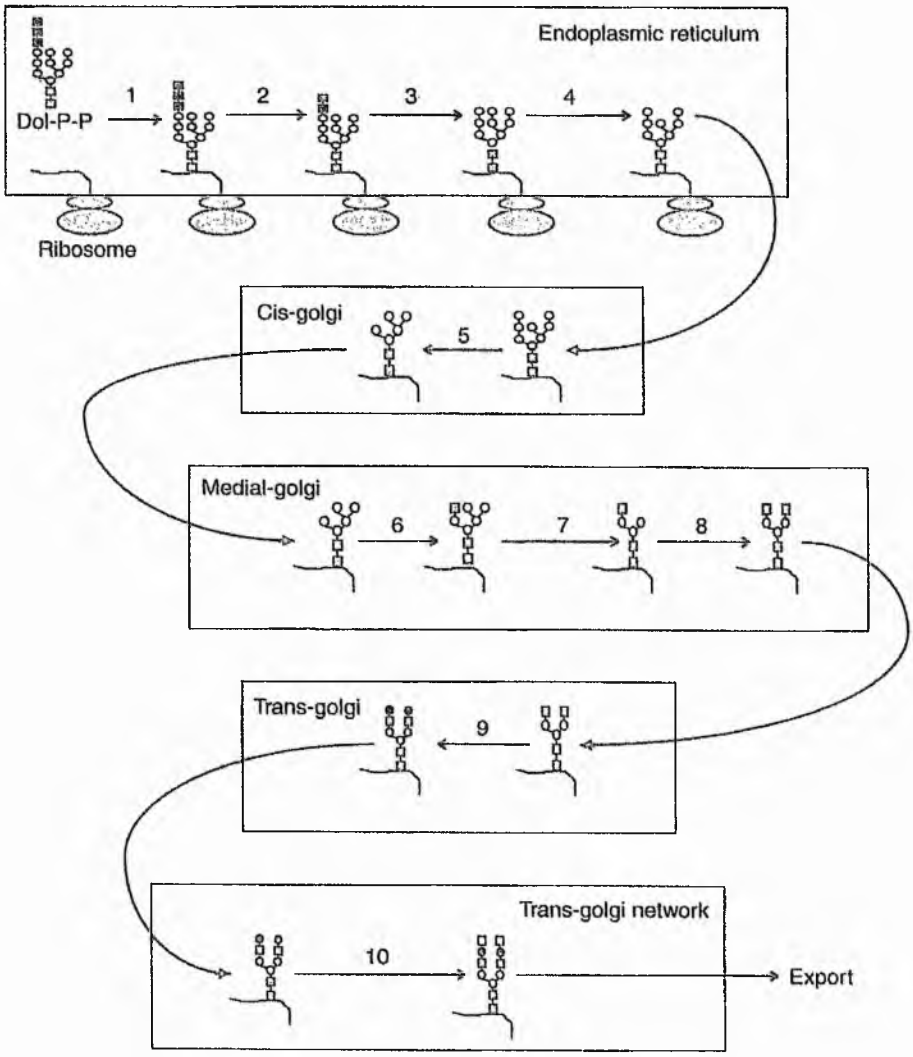


Figure 1.2. A representative pathway for the formation of a bi-antennary glycan chain (adapted from Keir et al, 1999). ■ = glucose; O = mannose; ⊡ = N-acetylglucosamine; ● = galactose; □ = N-acetyl neuraminic acid; Dol-P-P = dolichol pyrophosphate. Enzymatic steps shown are as follows:

1	Oligosaccharyltransferase
2,3	α-glucosidases
4,5	α2-mannosidases
6	GlcNAc-transferase I
7	α3 or α6-mannosidase II
8	GlcNAc-transferase II
	Core α6-fucosyltransferase
	GlcNAc-transferases III, IV, V and VI
9	β3 or β4-Gal-transferases (or β4-GalNAc transferase)
10	α2,3 or α2,6-sialyltransferases (or sulpho-transferases)

The product of this processing is the structure [Manα1-6(Manα1-3)Manα1-6](Manα1-3)Manβ1-4GlcNaAcβ1-4GlcNAcβ-Asn, i.e. ($Man_5GlcNAc_2$-R), which is the starting core for the synthesis of all complex and hybrid N-glycans.

The key enzyme for initiating this synthesis is N-acetylglucosaminyltransferase (GlcNAc-transferase) I, which adds the first N-acetylglucosamine (GlcNAc) unit to the core using uridine diphosphate GlcNAc (UDP-GlcNAc) as donor (Step 6). Then α-mannosidase II clips off further mannose units (Step 7), which allows GlcNAc-transferase II to begin extending the second antenna, again using UDP-GlcNAc as donor (Step 8). Also at this stage, fucose can be added by core α6-fucosyltransferase, and further GlcNAcs by GlcNAc-transferases III, IV, V and VI (GlcNAc-transferase III adding the "bisecting" GlcNAc to the middle mannose of the trimannosyl core). All these enzymes in steps 7 and 8 cannot proceed until GlcNAc-transferase I has initiated the sequence.

β3 or 4-galactosyltransferase then adds the galactose residues donated from UDP-galactose (or β4-GalNAc transferase adds GalNAc) (Step 9). Finally α(2,3) or α(2,6) sialyltransferase adds sialic acid transferred from CMP-N-acetylneuraminic acid (Step 10).

There are many crossroads during biosynthesis at which more than one enzyme competes for a common substrate. The route taken by the synthetic pathway at a competition point is mainly dictated by the relative activities of the competing enzymes (Schachter, 1991). Some glycosyl residues act as a 'stop signal': for instance the bisecting GlcNAc prevents the actions of α3/6-mannosidase II, core α6-fucosyltransferase and GlcNAc-transferases II, IV and V, so preventing further branching. However, the bisecting GlcNAc does not prevent the glycan chain from moving to the *trans* Golgi, with possible addition to the antenna of D-Gal, N-acetyl-D-galactosamine, sialic acid, sulphate, L-fucose or other residues. These points help to explain *how* microheterogeneity arises: *why* it should occur will be considered in later sections.

1.2.6.2. O-Glycan Biosynthesis

The synthesis of O-glycans starts in the cis Golgi, and does not involve dolichol derivatives. As for N-glycans, the synthetic paths are ordered rather than random i.e. certain key glycosyl residues divert the synthetic flux either away from or into a particular pathway. Many of the enzymes involved are common to both N- and O-glycan pathways. However, with O-glycans, no glycosidases are involved, but instead sugars are transferred individually from nucleotide sugars. There are eight possible core structures, which may be further elongated and terminated in many ways, to produce hundreds of possible O-glycan chains.

1.2.7. Functions of Glycan Chains

Some of the functions of glycans have already been alluded to in Section 1.5. These broadly fall into two main categories: structural or recognition functions, although these two cannot be totally separated.

1.2.7.1. Structural Functions

Some oligosaccharides are important in the physical maintenance of tissue structure, integrity and porosity, as seen in the heparan, dermatan and chondroitin chains of the proteoglycans in the formation of basement membranes and extracellular matrix. Related to this are protective or stabilizing roles. For instance, a coating of glycoconjugates around a whole cell can form a protective 'glycocalyx', and oligosaccharides on many glycoproteins can protect the polypeptide from the action of proteases or antibodies (Varki, 1993).

N- and O-linked glycans contribute overall to the physical properties of a protein, influencing its solubility, viscosity, hydrogen bonding, overall charge, degradation, stability, folding and conformation (Brockhausen, 1993). It is well accepted that the N-linked oligosaccharides of glycoproteins initiate the correct polypeptide folding in the rough ER, and subsequent maintenance of protein solubility and conformation. The involvement

begins as the nascent polypeptide is extruded into the ER, and the $Glc_3Man_9GlcNAcAc_2$ structure is transferred *en bloc* from the lipid-linked precursor, to an Asn group in the polypeptide, before folding is complete (Paulson, 1989). Many proteins that are incorrectly glycosylated fail to fold properly and are degraded. However, treatment with tunicamycin, which inhibits N-linked glycosylation, causes some proteins to have merely compromised biological activity, while others appear totally unaffected (Kornfeld and Kornfeld, 1985).

1.2.7.2. Recognition Functions

The remarkable diversity of carbohydrate complexes plays a pivotal role in determining the specificity of many biological recognition phenomena. Intracellular and intercellular trafficking – movement of molecules from one receptor to another – is often dependent on carbohydrate ligands (Varki, 1993). Cell adhesion molecules, as mentioned in Section 1.5, recognize carbohydrate groups. Since all cells are covered with a dense coating of sugars, oligosaccharides are critical determinants of 'cell-cell' interactions. During cell growth, development and differentiation there are continuous changes of cellular interactions mediated through cell-surface antigens on glycolipids and glycoproteins (Feizi, 1985). Fertilization in mammals depends on the binding of sperm to α-linked Gal residues of O-glycans on the surface of the egg (Miller et al, 1992).

As well as determining cell-cell and cell-molecule interactions within an organism, oligosaccharides are used by a variety of viruses, bacteria and other parasites to target host cells. Viruses often have carbohydrate-containing membrane lipids or glycoproteins on their surface, whose purpose may be to shield the virus from immune attack, or to interact with host cells or other viruses. Some of the surface proteins may be lectins or agglutinins recognizing specific host cell carbohydrates. For instance, the influenza viruses bind to residues containing terminal sialic acid (Alford et al, 1994). The *falciparum* malaria merozoite binds to sialic acid containing residues on host erythrocytes (Orlandi et

al, 1992). Bacteria have been shown to interact with carbohydrates of glycolipids (Lingwood, 1991) and mucins, and have glycosidases which act on mammalian glycoproteins.

Oligosaccharides also have important roles in host protection and immunity. As well as coatings to protect them from proteases, mentioned previously, addition of specific monosaccharides or modifications mask the sequences recognized by microorganisms, toxins or autoimmune antibodies. For example, the addition of a single O-acetylester to the 9-position of terminal sialic acid residues abrogates binding of the highly pathogenic influenza A viruses (Alford et al, 1994). The presence of terminal sialic acid on glycoproteins and cell surfaces masks recognition of βGal and βGalNAc residues by pathogens as well as endogenous macrophages. Sialic acid prevents rapid clearance from circulation by asialo- and asialogalacto-glycoprotein-specific receptors on hepatocytes and macrophages (Paulson, 1989). This could be seen as a form of defence mechanism, since sialic acid is not found in viral or bacterial glycans. The phenomenon also means that recombinant glycoproteins, such as tissue plasminogen activator and plasminogen, produced commercially for administration to humans or other animals, must be produced in mammalian cells or it will be cleared rapidly from the circulation. There are several other receptor mechanisms which recognize glycan determinants on circulating proteins, such as the hepatic fucose receptor, the mannose-6-phosphate receptors and the Man/GlcNAc receptor of the reticulo-endothelial system (Dahms et al, 1989; McFarlane, 1983).

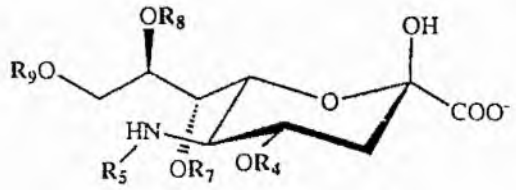
The glycosylation of the immunoglobulins, especially IgG, and autoimmune mechanisms will be described later. As well as the adaptive immune response, which in mammals is based on sophisticated gene rearrangement processes that yield a diversity of antibodies and cellular receptors, the more primitive innate immunity is based on recognition by carbohydrates, as a first line of defence (Gabius et al, 1997). There are a

number of molecules directed against common constituents of pathogenic organisms, distinguishing (hopefully) infectious non-self from harmless self (Janeway, 1992). Lectins, including acute-phase reactants such as C-reactive protein, are an important group in this class of molecules, and will be considered later.

Finally, a number of glycoproteins function as hormones. The pituitary hormones LH, FSH, GH and TSH contain N-glycans with differing distributions of terminal sialic acid and SO_4-4-GalNAc structures. Neither sialic acid nor sulphate are required for hormonal activity or receptor binding. Sialic acid appears to function in the prevention of rapid clearance of hormone from the body via the hepatic asialoglycoprotein receptor, and in this way may influence hormone potency (Thotakura et al, 1991). Sulphate increases hormone clearance by a specific hepatic receptor (Baenziger et al, 1992).

1.2.8. Sialic Acids

Although sialic acids are only one of the many monosaccharide components of glycoconjugates, a section is devoted to them because they are important with regards to this project for several reasons. They are widespread in nature and abundant constituents of plasma membranes, gangliosides, most secreted proteins and mucins, (Reutter et al, 1997). They are often in the peripheral position of oligosaccharide chains in glycoproteins and glycolipids, which makes them of prime importance in recognition and targeting roles. Thus they are involved in the modulation of many cellular processes, and in many aspects of cancer, infection and immunology. Moreover, they are the only monosaccharide in glycoconjugates to carry a charge, which has implications not only in their biological roles, but also in analysis of oligosaccharides, where charge differences can be exploited as in, for instance, HPLC and IEF.



R_5	$R_{4,7,8,9}$
$-CO-CH_3$ (N-acetylneuraminic acid)	-H (4,7,8,9)
$-CO-CH_2OH$ (N-glycolylneuraminic acid)	$-CO-CH_3$ (4,7,8,9)
-H (neuraminic acid; only stable in glycosidic bonds)	$-CO-CHOH-CH_3$ (9)
	$-CH_3$ (8)
	$-SO_3H$ (8)
	$-PO_3H_2$ (9)

Figure 1.3. The chemical structure of neuraminic acids. All natural derivatives of neuraminic acid share an elementary 9-carbon carboxylated structure. However several positions ($R_{4,5,7,8,9}$) can be substituted by different chemical residues as indicated in the table. More than 40 naturally occurring sialic acids have been identified so far (Reutter et al, 1997)

The term sialic acid is given to all acetylated derivatives of neuraminic acid, which share an elementary 9-carbon carboxylated skeleton (Figure 1.3). With many different substituents, the sialic acids are the most varied structurally of any group of natural monosaccharides (Varki, 1992). The most prominent member is N-acetylneuraminic acid (Neu5Ac), found in N-linked oligosaccharides including IgG. Sialic acid occurs most abundantly in vertebrates, but is found in most other types of living organisms except certain bacteria. They are derived in the cells from N-acetylglucosamine (GlcNAc) by a series of enzymatic reactions.

Five major biological functions have been attributed to the sialic acids (Crook, 1993; Powell and Varki, 1995).

1. Their large electronegative charge, with a pK value of about 2, under physiological conditions suggests a role in the binding and transport of positively charged molecules. The charge gives rise to attractive or repulsive forces between different cells, and provides an overall electronegative charge on cell surfaces. They are thus

involved in adhesion, aggregation and agglutination. They also function as recognition molecules for various cellular adhesion molecule such as the selectins, the sialoadhesins, CD22, myelin-associated glycoprotein (MAG) and neural cell adhesion molecule (N-CAM).

2. They are essential components of many cell-surface receptors for example the receptor for insulin. They are also involved in the modulation of amino acid transport in certain cells.
3. They are important for glycoprotein conformation, solubility, viscosity and charge. The grouping of glycoproteins on cell membranes is partly due to a mutual repulsion of their sialic acid residues, important for conferring rigidity to the cell.
4. They are an important part of many glycoprotein and glycolipid antigenic determinants, notably in the blood group antigens.
5. They protect glycoconjugates and cells from degradation, acting as shields to prevent recognition by clearing receptors. This is important in the removal from the circulation of glycoproteins and also cells such as platelets and erythrocytes. The removal of terminal sialic acid exposes galactose, which can be a recognition site for antibodies that bind and remove glycoproteins and cells via the reticulo-endothelial system.

They also protect proteins from degradation by steric hindrance of proteolytic action (Sjoberg et al, 1994) or by alteration of the biophysical environment. Rates of degradation of glycoconjugates are strongly influenced by the structure of their sialic acids: the half-lives of C_7, C_8, and C_9 *O*-acetylated sialic acids are markedly longer than that of unmodified Neu5Ac because their sialidases are longer acting (Schauer, 1991). Gastric mucus, a highly viscous gel containing many O-linked sialylated oligosaccharides, protects the stomach from self-digestion by secreted HCl (Bhaskar et al, 1992).

1.2.9. Evolution and Diversity of Glycans

1.2.9.1. Suggested Evolutionary Stages

Examination of the biosynthetic pathways and biological functions of oligosaccharides in different groups of organisms has produced a picture of the way in which they may have evolved (Drickamer and Taylor, 1998).

The sugars in mammalian oligosaccharides can be divided into two groups: (1) core sugars - e.g. GlcNAc, mannose, glucose - which establish the basic branching pattern, complemented by extensions – usually polylactosamine chains i.e. Gal-GlcNAc repeats; (2) A variety of terminal elaborations e.g. galactose, GalNAc, L-fucose, sialic acid and sulphates. In all eukaryotic cells, the initial N-linked carbohydrate structure that is transferred *en bloc* to proteins as they emerge in the ER consists of two GlcNAcs, nine mannoses and three glucoses. The core is then modified during passage through various luminal compartments, with glucose and some mannoses being removed, and then the terminal elaborations being added.

It is suggested that the core could be a primordial structure, since organisms such as yeast contain very large high mannose structures lacking mammalian-type elaborations, and has a much older role as a cell-wall constituent. The elaborations appear to have evolved more recently, extensively within the animal kingdom, and in a slightly different way in plants (Driouich et al, 1993). This theory is supported by the intracellular location of the glycosylation machinery: the circuitous route of adding a set of nine mannose residues and then taking them away suggests the grafting of a new pathway onto an old one.

O-linked oligosaccharides, with no *en bloc* transfer during their synthesis, can be regarded as comprising mainly terminal extensions, and it is suggested that they developed in parallel with the more recent part of the N-linked pathway.

In higher organisms, many of the terminal groups operate as recognition structures for a number of different receptors. Hepatic receptors for instance remove circulating asialoglycoproteins that bear terminal galactose residues, and glycoprotein hormones that bear terminal GalNAc-4-sulphate residues. Selectins and sialoadhesins interact with sialylated cell-surface ligands. All of these are useful only in multicellular organisms.

Recent evidence suggests that in the luminal compartments of the ER removal of glucose from the core structure indicates to the sorting mechanism that the synthesized protein is successfully folded, mediated by the chaperones calnexin and calreticulin (Fieldler and Simons, 1995). Some components of the sorting mechanism exist in yeasts but not in prokaryotes, suggesting that the structural role was followed first by this sorting role and then by the cell/ligand recognition role.

The effects the integral oligosaccharides have on a glycoprotein's properties, such as folding, stability etc., are thought to be a relatively recent development. Once the glycosylation machinery was in place, attached oligosaccharides inevitably became an essential part of the protein. It appears that the core sugars have importance in this role, probably due to their proximity to the peptide surface, while the terminal elaborations are distally located for interaction with other ligands.

This view helps to explain why sugar residues sometimes perform functions in protein structure that one would think could be accomplished by amino acids. The evolution of the glycosylation machinery gave rise adventitiously to protein modifications, which were subsequently selected for.

1.2.9.2. Diversity and Complexity

One of the striking things, mentioned already, about oligosaccharide chains is the diversity of glycosylation that is found at every level of biological organization. In many instances, this heterogeneity has no obvious function that benefits the organism (Gagneux

and Varki, 1999). The greatest diversity is found in the terminal elaborations where interaction with other ligands occurs. However, most pathogens have their own recognition molecules, such as lectins, which react to these terminal glycans and hence gain access to the host's biological processes. It has been proposed that multi-cellular organisms with long life cycles must constantly change in order to keep "a step ahead" of potential pathogens, which have much shorter life cycles and thus evolve faster. This has been named "The Red Queen Effect" (Van Valen, 1974), after the Red Queen's comment to Alice in *Through the Looking Glass* that "it takes all the running you can do just to stay in the same place".

The vertebrate immune system relies on somatic mechanisms for the generation of variation i.e. somatic recombination, hypermutation, gene conversion and clonal selection, which allows somatic vertebrate defence cells to evolve at rates comparable to those of microorganisms. However, allowing somatic cells to evolve at this rate, incurs the risk of uncontrolled proliferation (cancer) and misdirected immune reactions (autoimmune diseases) (Gagneux and Varki, 1999).

Other ways that glycans function to ward off pathogens have been proposed. Endogenous recognition in vertebrates is often through lectins which have poor single-site affinities (i.e. 'looseness of fit'), and will permit variation in ligand structure, whilst maintaining recognition (Weis and Drickamer, 1996). When the vertebrate makes a permitted variation pathogens are unable to recognize the modification, and so the vertebrate 'keeps ahead'. Similar processes may occur because of the inherent flexibility of glycosidic linkages in comparison to peptide bonds.

Some molecules have been proposed as 'decoy' structures. For example glycophorin A is a very complex membrane glycoprotein in erythrocytes. It does not appear essential to host functions, as individuals genetically lacking this protein suffer no apparent ill effects, but perhaps is a 'sink' for glycan-binding viruses. Viruses could be

'sopped up' by these non-nucleated cells which cannot serve as hosts for replication (Wybenga et al, 1996).

Glycoproteins generally exist as populations of glycosylated variants (glycoforms) of a single polypeptide, but why so many variants should exist is not well understood. IgG, for instance, has about 30 glycoforms. One theory is that this confers a 'herd immunity' to the protein: a pathogen recognizing a specific glycan cannot infect all members of a heterogeneous population (Gagneux and Varki, 1999). In the case of IgG, could it be that the IgG is 'offering' a variety of receptors to actually attract a wide-spectrum of pathogens with different receptor specificities? Immunoglobulins, of course, are proteins with capabilities of dealing with foreign invaders. More will be said about IgG in later sections.

1.3. Glycosylation Changes in Disease

Changes in glycosylation have been observed in many diseases and some aspects of some the main disease groups, with a few examples, will be looked at here, which may help towards understanding the disease mechanism of MS.

1.3.1. Congenital Disorders

Diseases due to a genetic deficiency of an enzyme in the biosynthetic pathway of N-glycans are rare, although more are coming to light as the disease symptoms and characteristics become more understood (Krasnewich and Gahl, 1997). Congenital dyserythropoietic anaemia type II (previously known as 'HEMPAS' – hereditary erythroblastic anaemia with multinuclearity and a positive acid serum lysis test) is caused by a deficiency of Manα:GlcNAc-transferase II (Fukada, 1990). Inclusion body cell disease (I-cell disease) is caused by deficiency of GlcNAc-1-phosphotransferase (Glickman et al, 1996). The carbohydrate-deficient glycoprotein syndromes (CDGS) – of which several types have been identified – manifest as multi-systemic diseases with major

nervous system involvement. Types Ia and Ib are related to deficiencies of phosphomannomutase and phosphomannose isomerase, respectively, while type II results from a deficiency of GlcNAc-transferase II (Jaeken et al, 1993). Types III, IV (Stibler et al, 1994) and V (Korner et al, 1998) have also been described. Diagnosis of the CDGS is often aided by the identification of the glycoforms of transferrin in CSF by isoelectric focusing (Keir et al, 1999). CSF is the better sample for the test as it contains a mixture of transferrin glycoforms having between 0 and 6 sialic acids, while transferrin in serum is less abundant and the glycoforms possess less than 3 sialic acids.

Another group of congenital diseases comprises the lysosomal storage diseases. These stem from deficiency of the enzymes required for glycosphingolipid catabolism within lysosomes, resulting in accumulation of undegraded substrate (Platt and Butters, 1998).

1.3.2. Cancer

The glycosylation of cells changes in cancer, with the type of change depending on the type of cancer as well as the underlying glycosylation programs of the cell type (Brockhausen, 1993). With the surface glycosylation of the tumour cell fundamentally altered, the interaction of the cell with other tissue cells, leukocytes, or extracellular ligands, including lectins, is substantially changed (Kim and Varki, 1997). The cell may therefore escape immune surveillance, leading to tumour progression and metastases (Yoshida et al, 1995). In general, the surface of cancer cells compared with normal cells has more branched glycans and is more highly sialylated. There may also be differences in fucosylation or galactosylation. The increase in branching is often at the GlcNAcβ(1-6) linkage on the Man(α1-6) arm of the trimannosyl core, so an increase in GlcNAc-transferase V (GnT V) may be a common feature in tumour progression.

Table 1.6. Structures of carbohydrate antigens (Kim et al, 1996).

Antigen	Structure
Tn	GalNAcα-Thr/Ser
Sialyl-Tn	NeuAc(α2-6)GalNAcα-Thr/Ser
T	Gal(β1-3)GalNAcα-Thr/Ser
i	Gal(β1-4)GlcNAc(β1-3)Galβ-R
Sialyl Le^a	NeuAc(α2-3)Gal(β1-3)Fuc(α1-4)GlcNAcβ-R
Le^x	Gal(β1-4)Fuc(α1-3)GlcNAcβ-R
Difucosyl Le^x	Gal(β1-4)Fuc(α1-3)GlcNAc(β1-3)Gal(β1-4)Fuc(α1-3)GlcNAcβ-R
Sialyl Le^x	NeuAc(α2-3)Gal(β1-4)Fuc(α1-3)GlcNAcβ-R
Sialyl Le^x (extended)	NeuAc(α2-3)Gal(β1-4)Fuc(α1-3)GlcNAc(β1-3)Gal(β1-4)Fuc(α1-3) GlcNAcβ-R
Le^y (short)	Fuc(α1-2)Gal(β1-4)Fuc(α1-3)GlcNAcβ-R
Le^y (extended)	Fuc(α1-2)Gal(β1-4)Fuc(α1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAcβ-R
Le^y (polymeric)	Fuc(α1-2)Gal(β1-4)Fuc(α1-3)GlcNAc(β1-3)Gal(β1-4)Fuc(α1-3) GlcNAcβ-R

Le: Lewis antigen

In cancer there may be expression of cell surface antigens that are not normally present in the particular tissue, but may occur elsewhere in the body or occur at a different period of growth or differentiation. They are often oncofetal antigens i.e. previously expressed in fetal life. A number of these antigens, usually O-linked oligosaccharides, have been found to be useful in determining the grade, metastatic potential or prognosis of the tumour (Muramatsu, 1993). These antigens exist in the core region of the oligosaccharides of normal cells, but are usually masked by additional sugar residues. Some of the commoner antigens are shown in Table 1.6. They tend to be more strongly expressed in tumour cells with either a poor prognosis or greater metastatic potential. In contrast, cells that normally express the ABH antigens tend to lose this expression when they become malignant.

The sialyl Tn antigen has been termed the 'pan-carcinoma antigen' and is expressed in 94% of adenocarcinomas of the colon, breast (84%), lung (non-small cell, 96%) and ovary (100%) (Kim et al, 1996). The Tn antigen in breast carcinoma is associated with poor prognosis and may be detected in tissue by *Helix pomatia* lectin binding (Leathem and Brooks, 1987). The Le^x structure is recognized by the *Lotus tetragonolobus* lectin, and indicates unfavorable prognosis in patients with carcinoma of the

bladder or colon (Nakagoe et al, 1993). Other antigens have been associated with various other diseases.

Cancer cells tend to produce increased mucins, which are high molecular weight, heavily O-glycosylated glycoproteins. MUC1 is a membrane type mucin highly expressed in breast and pancreas cancer cells. Other mucins have been associated with other cancer types (Kim et al, 1996).

As well as cell surface antigens, other cancer markers exist in serum or urine. These include carcino-embrionic antigen (CEA), CA-125, CA 15.3, CA 19.9, and CA 26 – all heavily glycosylated and containing blood group antigen related glyco-epitopes. Serum alpha-fetoprotein (AFP) shows differences in fucosylation in liver germ cell tumour compared to hepatomas when reacted with *Lens culinaris* agglutinin (LCA) (Aoyagi et al, 1998). Human chorionic gonadotropin (HCG) in urine from women with invasive hydatidiform mole has additional triantennary chains compared with urine from women in normal pregnancy. Abnormal biantennary chains are found in urine HCG from patients with choriocarcinoma or non-invasive mole (Kobata and Takeuchi, 1999) and can be distinguished by using a Datura stramonium agglutinin (DSA) column.

1.3.3. Infection

Some features of the recognition and interaction of pathogenic organisms with hosts' cells have already been mentioned in Sections 1.2.7 and 1.2.8. Glycosylation in relation to infection can be seen in two ways. Firstly from the point of view of the pathogen as a means of invading a host's cells, and secondly in relation to the host's defence mechanisms.

1.3.3.1. Pathogen Strategies

In this section, for simplicity, the term 'parasite' will refer to a non-viral or non-bacterial pathogen.

a. Parasites

As in mammalian cell-cell interactions, the parasite-host cell interaction is a complex step-wise process, involving multiple receptors and complementary ligands on both cell types (Ward, 1997). A primary step is the attachment of the organism. This can lead to colonization, as occurs with *Giardia lambda*-enterocyte interaction, lysis of the host cell, which is characteristic of *Entamoeba histolytica* infection, or invasion of the host cell, as with *Plasmodium falciparum*. These interactions are mediated by carbohydrate-binding proteins, which may be lectins or enzymes, on the parasite, and their specific carbohydrate ligands on the host cell (Figure 1.4). Host cell ligands may include glycoproteins, glycolipids or proteoglycans.

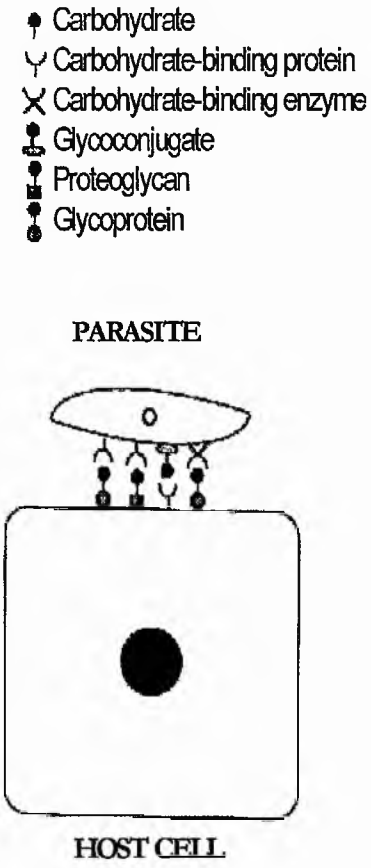


Figure 1.4. Attachment of parasites to host cells is mediated by carbohydrate-binding proteins and complementary ligands on both cell types. Various possibilities are shown (Ward, 1997).

Carbohydrates targeted by parasites include, primarily, sialic acids, GlcNAc, galactose, GalNAc, heparin and chondroitin sulphate. Sialic acid on host erythrocytes is believed to be involved in the adherence and invasion of erythrocytes by *Plasmodium falciparum*, and sialidase treatment of human erythrocytes leads to reduction of invasion (Hadley et al, 1986). In addition, human erythrocytes lacking the sialoglycoproteins glycophorin A and B, or the sialic component of these are resistant to invasion (Hadley et al, 1987). *Trypanosoma cruzi* protozoa also recognize terminal sialic acids on host glycoconjugates. After binding, they transfer host

sialic acids to their own surface by the coordinated activity of a neuraminidase and a sialyltransferase, which then shields the parasite from the host immune response and complement system (Tomlinson et al, 1994). A Gal/GalNAc-specific lectin of *Entamoeba histolytica* enables the trophozoites to adhere to host colonic cells (Burchard et al, 1993). This is followed by invasion leading to lysis of host tissue and dissemination of the parasite to extraintestinal sites.

b. Bacteria

The adherence of bacteria to host cells is largely by means of various bacterial lectins with specificity for particular carbohydrates. The lectins may interact with a range of receptors, enabling adherence to many tissues in diverse hosts. The presence of several lectin types on the same bacterium may allow adhesion to almost any cell type (Gilboa-Garber et al, 1997).

Some bacteria have preferential binding for a particular kind of cell. *Helicobacter pylori*, for instance, binds to the surface of gastric epithelial cells in the stomach using the Lewis^b antigens (Boren et al, 1993). However an adhesin binding to sialic acid has also been found in *H. pylori* (Evans et al, 1993). *Vibrio cholerae* bacteria produce the cholera toxin of which the B-subunit binds the glycosphingolipid GM_1, enabling the A-subunit to enter the host cell and activate cAMP production (Masco et al, 1991).

c. Viruses

Many viruses bind to glycoconjugates on hosts' cells by means of surface receptor proteins, such as haemagglutinin. Terminal sialic acid is often the target molecule (Reutter et al, 1997). The influenza A virus strain binds specifically to Neu-5-Ac α(2-6)Gal residues, whereas the B strain of the same virus is specific for to Neu-5-Ac α(2-3)Gal residues (Schauer et al, 1985), while the influenza C strain prefers Neu-5-Ac with 9-*O*-acetylation (Zimmer et al, 1994). For effective infection and replication, the virus needs a

second surface enzyme, sialidase, which attacks the protective mucus layer on mucosal cells (Corfield, 1992). Drugs have been developed which inhibit the viral sialidase (Von Itzstein et al, 1993).

The human immunodeficiency virus (HIV) has a major envelope glycoprotein (gp 160) which is unusually highly glycosylated, and much research has been focused on this structure (Fenouillet et al, 1994). Imino sugars such as N-butyldeoxynojirimycin (NB-DNJ) inhibit the processing enzymes α-glucosidases I and II, resulting in modification of N-glycans in the envelope glycoproteins, and reduction in infectivity of the virus (Fischer et al, 1995).

1.3.4. Host Defences

The vertebrate immune system can be divided into specific (adaptive) or aspecific (innate) means of defence, although the two systems are not separated. Most proteins in the defence systems are glycosylated, and awareness is growing of the importance of the attached sugars (Rudd at al, 1999).

1.3.4.1. The Adaptive Immune System

The adaptive immune system can be divided into humoral and cellular mechanisms, although the division is artificial, with interaction of molecules and cells. The adaptive response is based on sophisticated gene rearrangement processes which yield an enormous diversity of antibodies and cellular receptors (Gabius et al, 1997). The reasons for glycosylation of the components are wide-ranging. Some examples are: assisting with protein folding through interactions with calnexin; stabilizing protein structure (as in IgG); providing protease protection (as in IgA1); modulating the presentation of cell surface glycoproteins (e.g. cell adhesion molecules, CD2, CD48 and CD59), and providing potential recognition epitopes (as in IgG and IgM).

The immunoglobulins have a common Y-shaped structure, composed of two identical heavy chains (H) and two identical light chains (L) (Figure 1.5). The heavy chains are designated α, β, ε, γ or μ for the different immunoglobulin classes. Each heavy chain comprises 4 or 5 domains, while each light chain has 2 domains. The domains each consist of approximately 100 amino acids and contain, as a basic structural unit, the immunoglobulin fold. The tertiary structure of this is formed by two β-pleated sheets packed face to face and stabilized by disulphide bonds. All the immunoglobulins contain variable (V) or constant (C) domains that have many similarities but are all structurally different and vary in their primary sequence. (Rudd et al, 1999). Constant domains on the H chains contain conserved N-glycosylation sites. In addition an average of 30% of

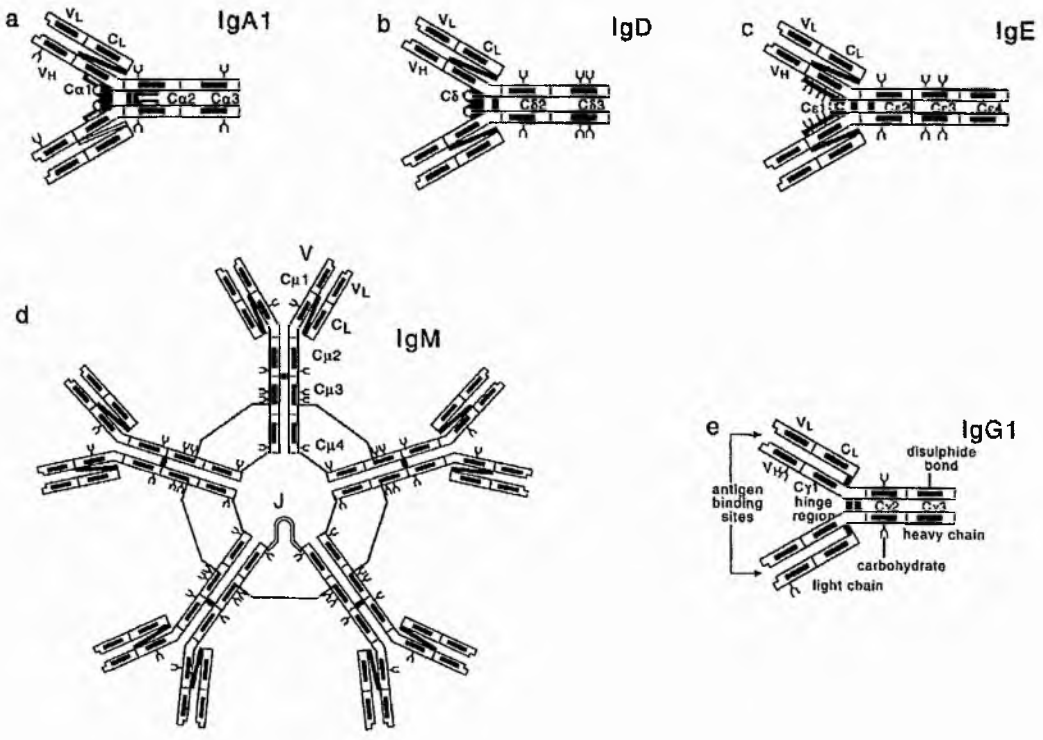


Figure 1.5. Examples of each of the five classes of immunoglobulins, showing regions of N-glycosylation (Y) and O-glycosylation (o) (Rudd et al, 1999).

serum IgA1 molecules (Mattu et al, 1998) and 40% of IgG molecules (Parekh et al, 1985) contain N-glycans in the variable Fab regions (V_H and V_L).

The occurrence of N-glycans is determined by the presence of the sequon AsnXaaSer/Thr (where Xaa is any amino acid other than Pro). The biosynthesis of the immunoglobulin glycans proceeds in the way described in Section 1.2.6. In the case of IgA or IgM, monomers are glycosylated and trimmed in the ER, and are then assembled into dimers or pentamers respectively before transfer to the Golgi. Overall it is not a functional requirement for an immunoglobulin domain to contain a glycan at any particular site, but the specific location does influence the processing of the glycan, and in some cases plays a critical role in the structure and function of the immunoglobulin (Rudd et al, 1999).

Conserved N-glycans in the Fc regions of IgG and IgA1 protect the peptides from proteases. IgA1 and IgD also contain a Ser/Pro/Thr rich hinge region between the Fc and Fab components, which is heavily O-glycosylated. This is also thought to protect the hinge region from protease attack, as well as extending the peptide chain and conferring rigidity to the structure through repulsion between the charged sialic acids. Only certain bacteria, using highly specific proteases, are able to cleave IgA1 at its extended hinge area. These include *Neisseria meningitides, Haemophilus influenzae, Streptococcus pneumonia* and *Neisseria gonorrhoeae* (Rudd et al,1999).

The quaternary structure of the immunoglobulins can restrict the accessibility of some of the glycosylation sites. The Fc sugars in IgG are contained in the interior of the CH2 domains, whereas in IgA1 the sugars are orientated away from the Fc surface and are fully exposed. Hence IgA1 sugars are larger, may be triantennary, and 90% are of the larger sialylated type, while in IgG only mono- and bi-antennary sugars exist, and only 15% are sialylated (Wormald et al, 1997). In IgM, N-glycans are contained in the center of the pentamer and are relatively inaccessible to proteins, including the enzymes in the ER

which process oligomannose structures to complex types. Thus the glycans found are oligomannose types. It is suggested these become clustered together when the IgM takes up a 'staple' conformation during interaction with an antigen, becoming exposed in a group above the surface of the molecule and available for complement activation (Wormald et al, 1991). This is an example of 'multiple presentation' which ensures that single molecules attached to 'self' proteins do not trigger defence mechanisms normally initiated only by 'non-self' pathogens.

As well as IgG, which will be discussed further in the next section, IgA glycosylation has been shown to change in disease. In IgA nephropathy, it is thought that IgA1 is over-sialylated in the hinge region, shown by increased binding to *Samubus nigra* agglutinin (SNA), which binds preferentially to NeuAc(α2-6)Gal (Baharaki et al, 1996).

1.3.4.2. The Oligosaccharides of IgG

a. IgG Structure

In human IgG two conserved bi-antennary oligosaccharide chains are attached to Asn 297 amino acids in each conserved $C_\gamma 2$ domain in the F_c region. These normally impinge towards the inside of the F_c regions, and interact with each other to stabilize the whole molecule (Figure 1.6). Non-conserved oligosaccharide chains occur in the F_{ab} region, and their frequency and location varies with the presence of Asn-X-Ser/Thr sites (where X is any amino acid apart from Pro). An immunoglobulin domain and a fully extended N-linked complex oligosaccharide are of roughly similar sizes (Keusch et al, 1996; Parekh et al, 1985). The largest glycan chain that is found in IgG is shown in Figure 1.7. Any of the residues underlined may be missing, thus conferring heterogeneity to the molecule.

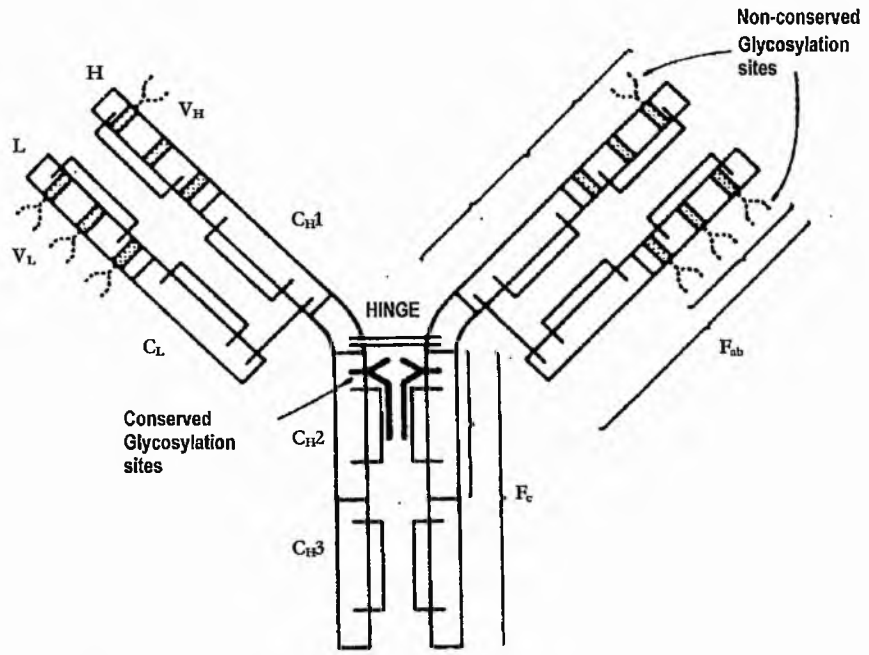


Figure 1.6. The structure of immnoglobulin G showing positions of the conserved and non-conserved oligosaccharide chains (Parekh et al, 1985).

b. Modulation of Behavior

Some ways in which glycans modulate the behavior of IgG and other immunoglobulins have been discussed, and more examples will be given here. The carbohydrate components are necessary for some, but not all, functions of IgG. If carbohydrate is removed from IgG - either by glycosidase digestion or by treating IgG secreting cells with tunicamycin, which inhibits glycosylation – the molecule loses its ability to bind to F_c receptors on macrophages (Dwek, 1995). It also has three times less

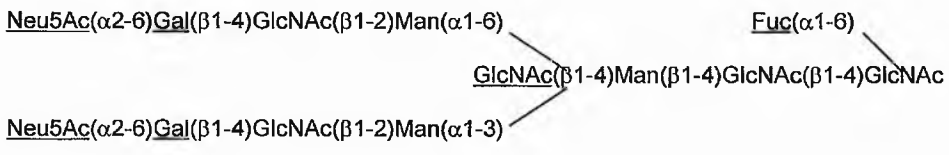


Figure 1.7. The largest N-linked sugar chain of human IgG. Residues underlined may be absent (Kobata, 1994).

affinity for the complement component $C1_q$ (Tsuchiya et al. 1988). But its antigen binding properties are not affected, suggesting that the F_{ab} glycans are not involved in specific immunity. However the antigen-antibody complexes formed failed to be eliminated from the circulation.

As already mentioned, oligosaccharides of IgG help stabilize the molecule and shield it from protease attack. The F_c glycans are particularly hidden (Figure 1.8). When terminal sugars are absent from these, the conformation of the IgG molecule may change (Rudd et al, 1999) and also the glycans may become less hidden and available for binding by lectins or other molecules (Figure 1.9).

In comparison to F_{ab}, F_c glycans have been found to contain: (i) lower levels of bisecting GlcNAc; (ii) lower levels of galactose; (iii) higher than expected levels of α(1-6)

Figure 1.8. Molecular model of IgG1 based on the crystal structures of IgG F_{ab} and IgG F_c. F_{ab} glycosylation sites are in the hypervariable region and are occupied approximately 40% of the time. Conserved glycosylation sites are in the F_c region and are normally shielded by the CH2 domains. (Carbohydrates are shown in blue, light chains in green, heavy chains in red and disulphide bonds in yellow). From Dwek et al, 1995.

arm galactose compared to that on the α(1-3) arm; (iv) no α(1-6) arm sialylation (Wormald et al, 1997). NMR relaxation was measured to compare the mobility of the glycans. In the Fc region the mobility depends directly on the primary sequence of the glycans: glycans with galactose on the α(1-6) arm have relaxation properties very similar to those of the unglycosylated protein backbone i.e. they have no independent motion. However glycans lacking galactose had relaxation rates 30 times slower than the peptide, i.e. much greater mobility. This confirms that the glycans lacking galactose do not interact with the protein, which would result in exposure of the previously covered peptide

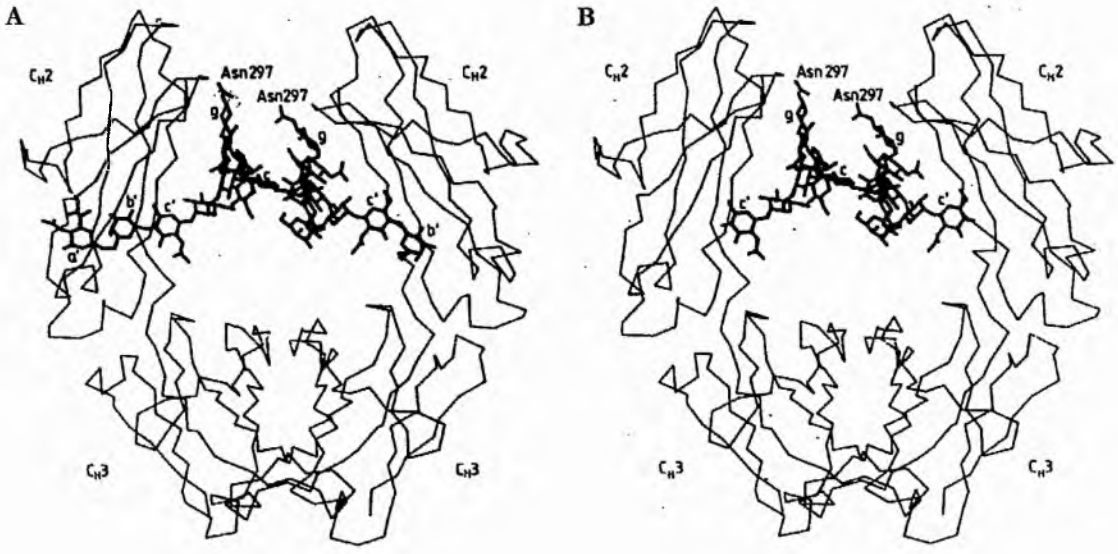


Figure 1.9. Refined structures at 2.8 Å of rabbit F_c fragment showing the two carbohydrate chains, each attached at Asn 297 (Parekh et al, 1985). **A** is a maximally glycosylated fragment: the α(1-3) arm of the left-hand chain is always devoid of galactose and interacts through its β(1-2) linked GlcNAc residue (c) with the Manβ(1-4) GlcNAc segment of the opposing oligosaccharide chain. The α(1-3) arm of the right chain extends outwards between the domains with no apparent steric constraints on its length; a Neu5Ac unit (a') is shown on one α(1-6) arm only (left). The electron density for this unit is weak, and experimentally no disialylated chains occur on the Fc. **B** is the same F_c fragment containing oligosaccharides devoid of galactose and sialic acid on each of the α(1-6) arms. Since these residues in normal IgG are in contact with the surface of the protein, their absence could make the IgG 'sticky' by creating a lectin-like activity.
Key: c: GlcNAc, g: core GlcNAc - on the α(1-3) arm; a': sialic acid, b': galactose, c': GlcNAc - on the α(1-6) arm.

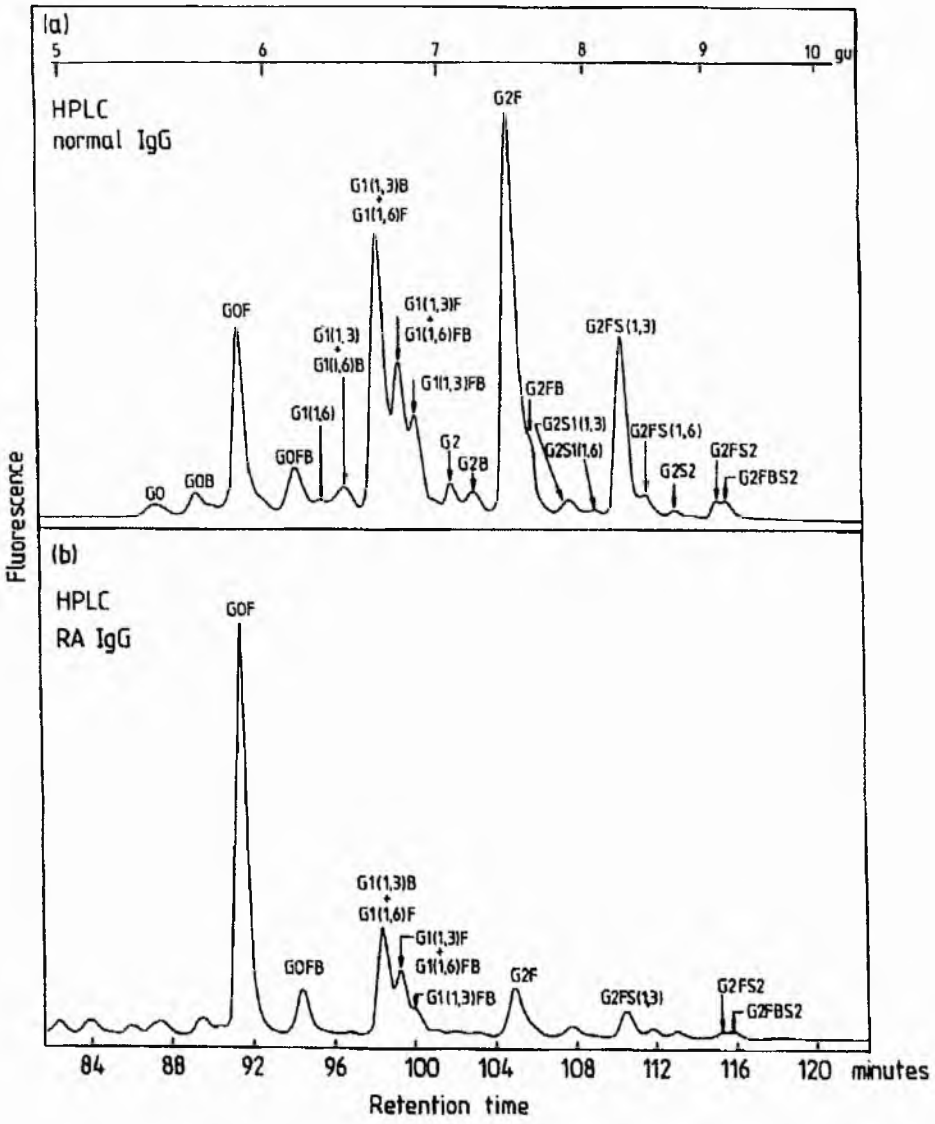


Figure 1.10. Normal-phase HPLC separation of total IgG glycans from (a) normal and (b) RA serum (Guile et al, 1996). All glycans, released from IgG by hydrazinolysis, are biantennary. G = galactose (0, 1 or 2 per glycan); F =fucose; S = sialic acid (0, 1 or 2 per glycan); B = bisecting GlcNAc. Peaks were assigned glucose unit values (gu) by comparison with a standard dextran ladder. The percentage G0 sugars, calculated from the peak areas, was 51.8% in RA IgG compared with 15.2 % in normal serum IgG

surface, as well as making the glycans more accessible (Wormald et al, 1997).

c. IgG Heterogeneity

Human IgG contains an average of 2.4 glycans per molecule (Youings et al, 1996), with 2 conserved glycans in the Fc, while Fab glycans are present 40% of the time (Wormald et al, 1995). Thus a variety of glycoforms are possible. HPLC analysis of IgG

has shown at least 24 different glycan structures, although three varieties on Fc, and two on Fab, predominate (Wormald et al, 1997). While variable heterogeneity has been found in IgG paraproteins (Farook et al, 1997) with overall increased sialylation (Fleming et al, 1998), the pattern of heterogeneity in serum IgG is surprisingly constant from one normal person to another (Mizuochi et al, 1982). For instance, HPLC of serum IgG from normal samples consistently gives a similar trace, as shown in Figure 1.10a, while IgG from rheumatoid arthritis patients gives a pattern as shown in Figure 1.10b, with larger galactose deficient (G0) peaks (Guile et al, 1996).

Remission of RA temporarily occurs during pregnancy, with a decrease in the proportion of IgG Gal 0. This is thought to be related to suppression of the mother's immune system so that the growing fetus is not rejected. Remission of MS during pregnancy also occurs. This phenomenon provides evidence that these diseases have an autoimmune basis. The percentage of IgG Gal 0 also distinctly increases with age (Shikata et al, 1998; Yamada et al, 1997) with the quantity of agalactosyl glycoforms being lower in females than in males.

1.3.4.3. Non-Adaptive Defence Mechanisms

The adaptive immune system is not sufficient to protect mammals at all stages of development. Innate, non-adaptive mechanisms are vital in the first 18 months of human life and as a first line of defence where there are low levels of antibody present (Gabius et al, 1997). Body fluids contain a variety of proteins which have non-specific anti-bacterial, anti-viral or anti-parasite activities. These include the complement proteins, lysozyme, acute phase proteins, cytokines and other regulatory proteins. Lectins are also an important group of animal defence molecules, and they may function as agglutinins to enhance phagocytosis, as opsonins, complement activators, cell recognition molecules and as initiators of mediator release.

Homologous mechanisms of innate immunity have been found in invertebrates and vertebrates (Marchalonis and Schluter, 1990). For example, the mammalian acute-phase reactants, pentraxins, and certain tunicate or horseshoe crab lectins are considered to belong to an ancient group of recognition molecules. In plants, also, some lectins, owing for instance to their toxicity, appear to serve as defence mechanisms. Some members of the lectin family in humans will be briefly looked at here.

The pentraxins, C-reactive protein (CRP) and serum amyloid P component share significant sequence homology indicating a common reactivity during the acute-phase response, although often only one of the pentraxins behaves as a reactant for the targeted species (Ballou and Kushner 1992). CRP acts as an opsonin for bacteria, with calcium-dependent binding to galactosyl residues, rendering them susceptible to phagocytosis (Köttgen et al, 1992). Amyloid P component, in the presence of physiological levels of calcium, binds to various ligands including chromatin, histones, heparan sulphate, mannose-6-phosphate, 3'-sulphated derivatives of glucuronic acid, galactose, and GalNAc (Tennant and Pepys, 1994). This binding activity aids in the clearance of nuclear and lysosomal material from necrotic areas during inflammation.

The collectins are a group of calcium-dependent (C-type) lectins which contain collagen-like domains. In serum collectins, the recognition domain (lectin) is linked to a potential effector mechanism resembling the organization of the first complement component, C1q (Malhotra et al, 1992). To fulfil their role of binding avidly to sugar epitopes on the surface of pathogens, serum mannose-binding proteins cluster their recognition domains. They form a triple helix with their collagenous tails, with further aggregation of three to six trimers, each tail holding a lectin, so that the arrangement resembles a bunch of flowers with the collagen tails as stalks and the lectins as flower heads. Each carbohydrate-binding domain in a trimer is separated from the others by 53 Å, which is optimum for binding to the widely spaced sugars on the surface of bacteria,

yeasts and other parasites (Weis and Drickamer, 1994), and also possibly to tumour cells that have aberrant glycosylation patterns (Ohta and Kawasaki, 1994).

Selectins are C-type lectins involved in the rolling of leukocytes and the targeting, homing and activation of lymphocytes (Zanetta, 1997). Each selectin, as well as a lectin-like domain, comprises an epidermal growth-factor-like domain, a number of complement-regulatory domains and a trans-membrane polypeptide. They exist on the surface of endothelial cells (i.e. E-selectins), on platelets (P-selectins) and on leukocytes (L-selectins) and recognize ligands on target cells by means of carbohydrate groups, notably sialyl Le^x and sialyl Le^a (Phillips et al, 1990). Other proteins involved in leukocyte adhesion and extravasation include the integrins and cell adhesion molecules which are members of the immunoglobulin superfamily – principally ICAM-1, ICAM-2, and V-CAM (Springer, 1990), and the neural cell adhesion molecule (N-CAM).

The cerebellar soluble lectins (CSL) are calcium-independent mannose-binding lectins of varying sizes, depending on the brain cell type. They recognize preferentially $Man_6GlcNAc_2$ N-glycans, but also hybrid type N-glycans sulphated and fucosylated on the core GlcNAc, and heparin. These lectins are important for both cell adhesion, by forming bridges between surface glycans on neighbouring cells, and for signal generation, by causing clustering of ligands at the surface of a cell.

Apart from lectins, there are other regulatory proteins involved in host defence through carbohydrate recognition, which themselves may be glycosylated. Cytokines are locally produced hormones that alert the innate and specific immune systems, and include the interleukins, interferons and cell colony stimulating factors. They are low molecular weight proteins (15-20 kDa) produced by leukocytes and other cells in low concentrations (10^{-15} M). They are highly potent and have multiple effects on the growth and differentiation of a number of cell types, combining with small numbers of high affinity cell surface receptors and producing changes in the pattern of RNA and protein synthesis

(Roitt, 1994a). Thus they are mediators in cell growth and differentiation, immunity and inflammation.

Most cytokines are glycosylated, with one or more glycosylation sites, and hence occur as populations of glycoforms. It is thought that glycosylation acts as a further control mechanism on their interactions with various proteins, such as the proteases, targeting them to specific cellular receptors and matrix binding sites (Van den Steen et al, 1998). In the matrix protease cascade (see next section) glycosylation is thought to be an additional level of control of amplification. N-linked sugars always have a down-modulating effect on enzymes and cytokines; O-linked sugars may be up- or down-modulating (Opdenakker et al, 1995). In stress situations of the host, in which N-linked glycosylation is less efficient, the activities of the cytokines and enzymes are increased which results in an increased metabolism and substrate turnover.

1.3.5. Autoimmune Disease

Most chronic inflammatory diseases have an autoimmune basis; therefore glycosylation plays a key role in these diseases since it is fundamental to the operations of molecules involved in the inflammatory response. These include the selectins; collectins and other lectins already described, as well as complement and various control proteins including cytokines. A number of inflammatory diseases are associated with a deficiency of terminal galactose on the oligosaccharides present on the F_c part of IgG in serum (Gal 0 or agalactosyl IgG). These include rheumatoid arthritis (RA), osteoarthritis, systemic lupus erythematosus, Crohn's disease and tuberculosis (Roitt et al, 1988; Tomana et al, 1988; Rook et al, 1989; Furukawa and Kobata, 1991). The reduction in terminal galactose appears to be due to reduced galactosyltransferase activity towards asialo-agalacto-IgG during synthesis in the B-cells (Axford at al, 1987), which might be due to a lowered affinity for UDP-galactose.

The lack of terminal galactose residues on IgG could itself be the cause of inflammation in RA and other diseases, through activation of the complement system, as follows. The absence of terminal sugars results in decreased protein-oligosaccharide interaction in the Fc, and the sugars are displaced out of the CH_2 domains. Thus exposed, they are prone to binding by endogenous lectins such as mannose binding lectin (MBL), which will then activate complement (Malhotra et al, 1995). This provides an additional route to complement activation to the classical pathways involving IgG antigen-antibody complexes or IgG rheumatoid factor (RF) complexes (Rudd et al, 1999).

In autoimmune diseases there is a breakdown in the mechanisms that control self-tolerance (Roitt, 1994b). In essence, the body produces antibodies against 'self' antigens, as opposed to 'non-self', e.g. an invasive pathogen. It is usually thought that this occurs after a foreign organism, such as a virus or bacteria, has invoked an immune response in a host which produces polyclonal antibodies against the organism. The antibodies are directed against a particular sequence of molecules, the antigen, which may be amino acids or carbohydrates. Subsequently, these antibodies recognize this same, or similar, antigen in the host, even though it is 'self' and poses no threat. Continuous immune response against 'self' antigens causes a chronic inflammatory response and chronic disease.

The role of autoimmunity in many disorders is not clearly defined, but a general model of the mechanism has been suggested, termed the Remnant Epitopes Generate Autoimmunity (REGA) model (Opdenakker and Van Damme, 1994), in which the cytokine production of matrix proteases is the basis of the disease (Figure 1.11). The regulation of extracellular proteolysis is controlled by a balance of inflammation-producing cytokines, which induce production of proteolytic glycoprotein enzymes (proteases) and disease-limiting cytokines, which induce protease inhibitors, in a cascade

system similar to the enzyme cascade in blood clotting. The system is triggered by an aetiological agent which could be viral or bacterial.

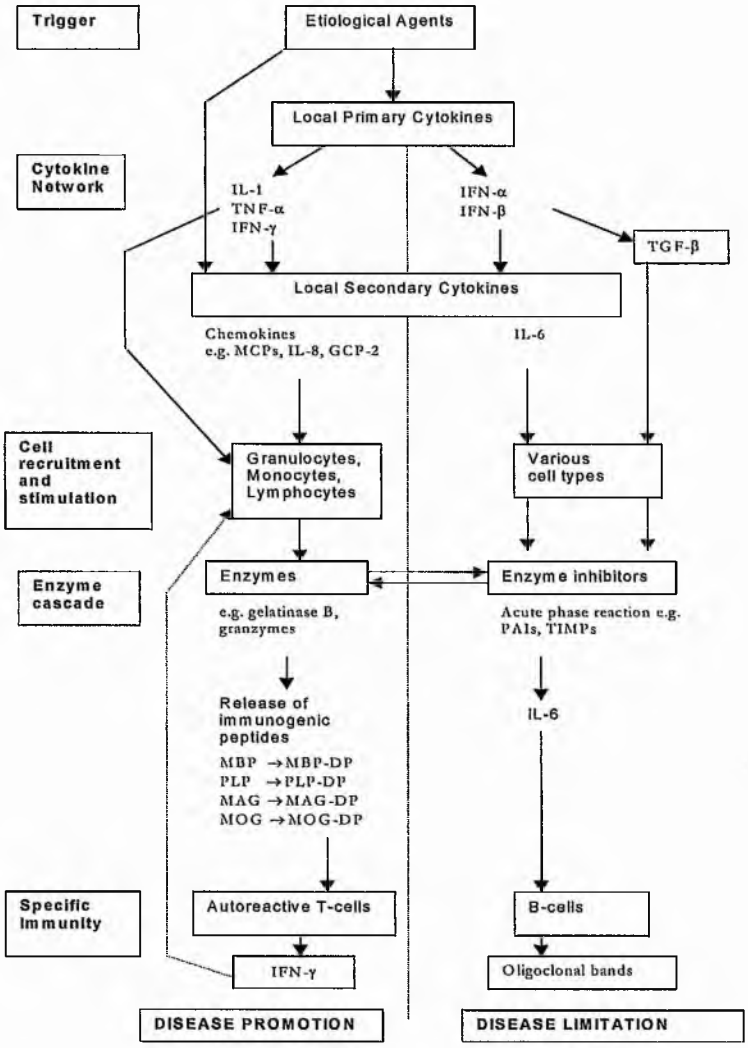


Figure 1.11. Cytokines and proteases in autoimmunity (Dubois et al, 1999). IL = Interleukin; TNF = Tumour necrosis factor; IFN = Interferon; TGF = Transforming growth factor; MCPs = Monocyte chemotactic proteins; GCP = Granulocyte chemotactic protein; MBP = myelin basic protein; PLP = Proteolipid protein; MAG = Myelin-associated glycoprotein; MOG = Myelin oligodendrocyte glycoprotein; PAIs = Plasminogen activator inhibitors; TIMPs = Tissue inhibitors of metalloproteases. DP = Degradation products.

One of the effects of the cytokine network and the matrix proteolytic enzymes is the degradation of matrix components such as collagen in connective tissue or myelin in

nerve tissue, so as to allow chemoattracted leukocytes to reach the inflammatory focus. In the case of chronic inflammation or autoimmune disease, matrix components are constantly degraded into small peptides (with the same sequence but perhaps another conformation), which contain epitopes that stimulate autoreactive T-cells (Dubois et al, 1999). In multiple sclerosis, the destruction is directed towards myelin components such as myelin basic protein (MBP), proteolipid protein (PLP), myelin-associated glycoprotein (MAG) and myelin oligodendrocyte glycoprotein (MOG) and results in different degradation products (DP). Autoreactive T-cells produce γ-interferon (IFN-γ) which further activates the inflammatory reaction. Oligoclonal immunoglobulins might assist in the elimination of specific antigens and, when complexed with antigens in the central nervous system (CNS) activate complement-mediated inflammation (Opdenakker and Van Damme, 1994).

1.4. Multiple Sclerosis

1.4.1. Occurrence

Multiple sclerosis (MS) is a debilitating autoimmune disease of the central nervous system (CNS) with demyelination – i.e. destruction of the myelin sheath that surrounds the axons – being the major pathological feature (Bannister, 1992). It is predominantly a disease of young adults, with a peak age of onset around 30 years, and is more common in women than men. It is most prevalent in Northern Europe, the Northern states of the USA, New Zealand and Tasmania, with an incidence of about 1 in 1000 (Figure 1.12). It is rare in tropical and sub-tropical countries and never seen in gypsies, Eskimos, Bantus, or native North and South Americans. There is a low incidence in Asia, but when it occurs the disease is often exceptionally severe. (Matthews, 1978).

1.4.2. Genetic factors

The geographical distribution is thought to result as much from genetic as from environmental factors (Ebers and Sadovnick, 1993). There is a strong association with the HLA-DR2 histocompatibility antigen and a slightly less obvious one with HLA-A3, -B7, -DW2 and -DQw1. In Japan the HLA-DR4 antigen is also common (Fukuzawa et al,

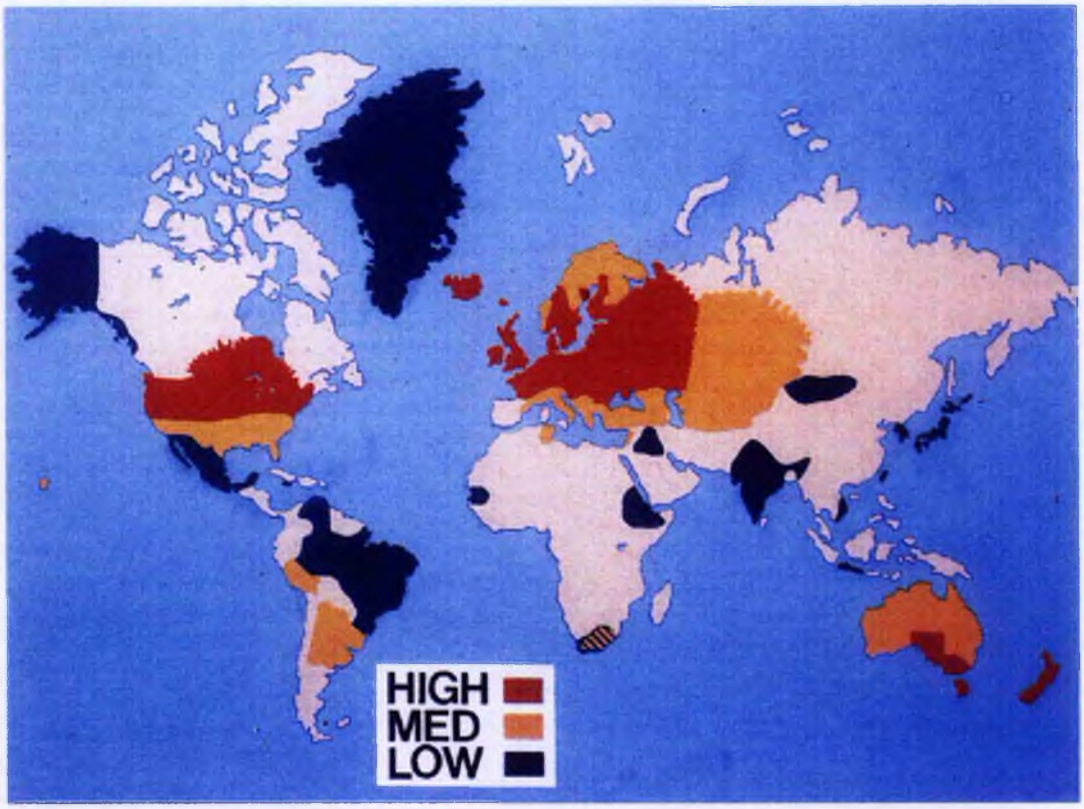


Figure 1.12. World incidence of multiple sclerosis. Pink areas denote no data available.

1998). It has been suggested that the HLA types associated with MS are characteristically Scandinavian, that the disease originated in this area and has been carried around the world by emigrants such as the Vikings (Adams, 1989).

1.4.3. Aetiological Agent

While it is generally accepted today that MS is an autoimmune disease, with a strong genetic influence, there have been many suggestions of the causative agent. It has been proposed that sunlight, especially in the winter, protects against the disease (Acheson

et al, 1960). Dietary deficiency has been suspected, particularly of polyunsaturated fatty acids (PUFA), which is required for synthesis of myelin. But a high polyunsaturated diet has been explored in MS and does not influence the rate of clinical deterioration (Bates et al, 1978). Coeliac disease has also been suspected, but studies on MS patients show normal histology of the small intestine (Bateson et al, 1979).

A number of infective agents have been implicated. Emigrants from cold to tropical climates have a higher incidence of MS than native born, unless they emigrated as children. Individuals born in a cold country from parents who emigrated from tropical parts have the same high incidence as the indigenous population. Hence it has been proposed that MS is a disease contracted in late childhood, manifesting itself some 10 years later (Elian and Dean, 1987). The low incidence of MS in hotter climates has been explained by supposing that children become exposed early to a variety of infective conditions (particularly enteric viruses) in hot climates and develop immunity to them at an early age before the critical age at which MS begins. There is little substantial evidence for these theories.

MS patients exhibit raised antibody levels to a wide range of viruses, including measles, rubella and herpes zoster (Reiber et al, 1998), Borna disease virus (Deuschle et al, 1998) and Epstein-Barr virus (Rand et al, 1998). Most investigators in the MS field surmise that viral infection (not necessarily a specific virus) or injury triggers the initial events in MS. Subsequently, an autoimmune response amplifies the process into a major relapsing disease. Possible mechanisms for this process will be discussed later.

1.4.4. Symptoms and Course of Disease

There are several patterns of presentation of MS and subsequent course (Walton, 1993; Bannister, 1992). Most commonly, MS presents as the relapsing-remitting (RR) form. There may be a single focal lesion, or several different lesions. Often the first

symptom is unilateral optic neuritis; other symptoms may be double vision, or numbness or weakness in some part of the body such as a limb or both lower limbs, or one side of the face. The initial symptoms then diminish over a period of weeks or months and then either disappear or leave behind a residual disability, but return later after a period of stability. Twenty percent of the MS population have a benign form of the disease in which symptoms show little or no progression after the initial attack. Primary-progressive (PP) MS is characterised by an insidious and gradual clinical decline with no distinct remissions. The symptoms are usually predominantly spinal, with progressive weakness of one or both lower limbs. Secondary-progressive (SP) MS begins with a RR course followed later by a PP course (Jongen et al, 1997), while a progressive-relapsing (PR) form takes a progressive path punctuated by acute attacks. In a typical advanced case, the patient becomes bedridden with severe paraplegia, staccato speech with slurring of syllables. There is likely to be incontinence, and possibly loss of emotional control.

1.4.5. Myelin Structure and Myelination

Myelin is a complex membranous structure around axons in the central nervous system (CNS) and peripheral nervous system (PNS) (Figure 1.13). It is made by the wrapping of processes of oligodendrocytes (in the CNS) or Schwann cells (in the PNS) (Adams, 1989). Its essential molecular structure is a lipid bilayer with protein connecting struts. The myelin sheath is essential for effective propagation of nerve impulses, and myelin abnormalities cause severe disorders in man, as seen in multiple sclerosis and Guillain-Barré syndrome.The protein and glycoprotein composition of myelin in the CNS and PNS is fairly well known. The most abundant myelin proteins of the CNS are myelin basic protein (MBP), the proteolipid proteins PLP and DM-20 and the Wolfgram proteins. The major glycoprotein of the CNS is myelin-associated glycoprotein (MAG). It is a member of the immunoglobulin superfamily endowed with 8 or 9 N-glycosylation sites.

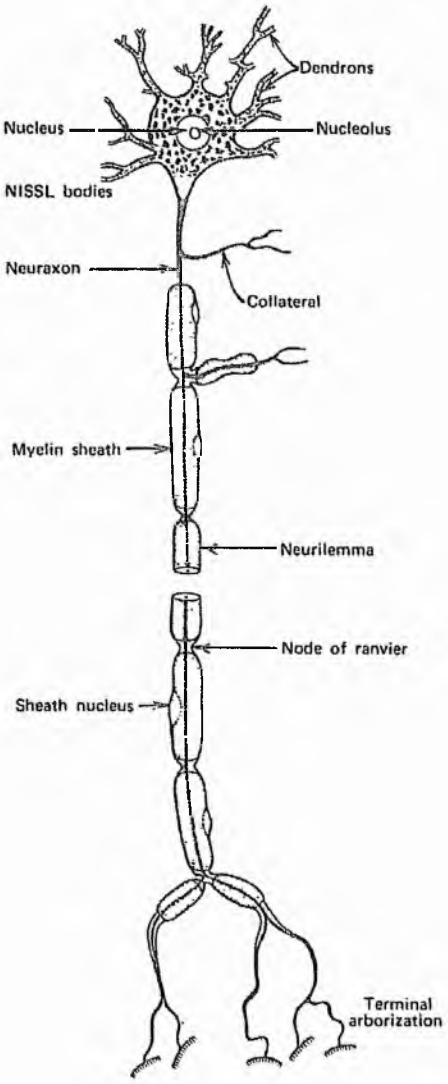


Figure 1.13. A neurone (Smith, 1992)

Some of the N-linked chains have the epitope HNK-1, which is glucuronic acid-3-sulphate (Burger et al, 1992).

Minor glycoproteins have been detected in the CNS myelin, but not characterised, which interact with an endogenous lectin, cerebellar soluble lectin (CSL). This mannose-binding, calcium-independent lectin stabilises protein structure by serving as a bridging molecule between glycoprotein glycans on the surface of the processes of myelin forming cells. Another minor glycoprotein is myelin-oligodendrocyte glycoprotein (MOG), which belongs to the immunoglobulin superfamily and has a single N-glycosylation site with an HNK-1 reacting glycan.

The metabolic stability (or speed of turnover) of myelin has been studied. Myelin lipids have a slow turnover characterised by metabolic stability, although some constituents, such as phosphatidyl choline and phosphatidyl inositide, turn over faster (Benjamins and Smith, 1984). Myelin proteins turn over much more rapidly (20-40 days) than the 'slow-turnover' myelin lipids (Smith and Hasinoff, 1971).

The oligodendrocyte is responsible for myelination in the CNS. Whereas the PNS is largely myelinated by birth in most species, the brain is slowly myelinated in the

months and years following birth (Adams, 1989). An oligodendrocyte is thought to myelinate 30 to 40 CNS internodes by a complex series of extrusions around the axon. The mature myelin sheath is maintained by these cells, and damage to the cells causes myelin degeneration. Myelination is controlled by thyroxine. Myelin development is at risk when malnutrition occurs in early life, and myelin defects may persist even though nutrition is restored.

1.4.6. Demyelination

Although an illness resembling MS was described in the Middle Ages, the disease was not formally recognized until the nineteenth century, when it was seen that selective damage to the myelin sheath occurs (Charcot, 1868). The myelin-oligodendrocyte system only is damaged; the axon and nerve cell remain intact (although the axon may be damaged at a later stage).

Demyelination of areas of the brain occurs as 'plaques' (Adams, 1989). These can be visualized by magnetic resonance imaging (MRI), where the demyelination appears as lightened regions of high signal (Figure 1.14). Gadolinium, a paramagnetic contrast agent, can be given intravenously, which will cross the non-intact blood-brain barrier, and enhance the image of some plaques in MS. Over 95% of patients with clinically definite

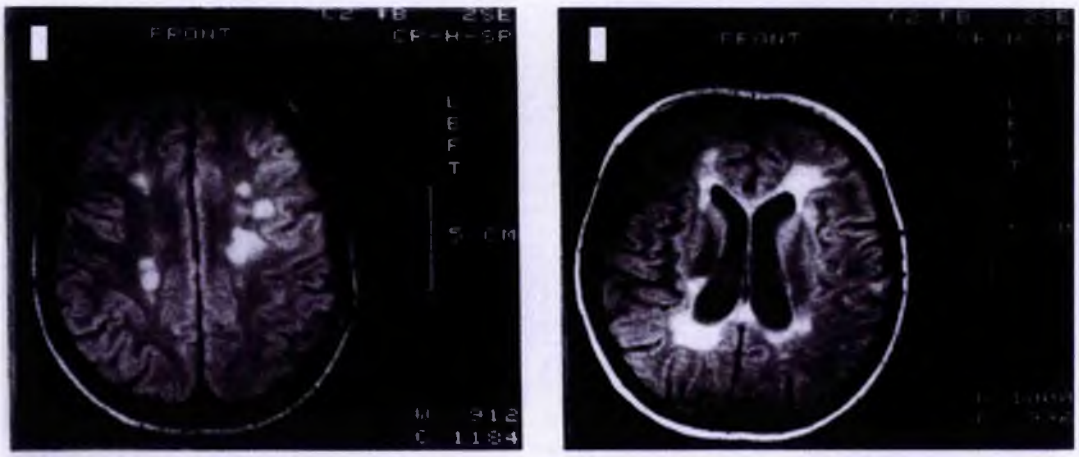


Figure 1.14. MRI brain scans of clinically definite progressive MS, showing (A) multiple 'high-signal' lesions in hemispherical white matter and (B) abnormal periventricular changes.

MS show white matter lesions, but similar lesions also occur in acute disseminated encephalomyelitis, cerebral vasculitis and sarcoidosis. Demyelination plaques seem to arise in the vicinity of the ventricles, around periventricular veins, with further plaques spreading into the brain. This seems to suggest that the early lesion is caused by something originating in the blood rather than the CSF. It is thought that the formation of plaques starts with the infiltration of T cells, B cells and immunoglobulins through the wall of small draining subependymal veins near the ventricular surface. Demyelination appears to spread outward from here, perhaps relayed by immunoglobulins from the ventricles (Zanetta, 1996). In the plaques, myelin is decompacted and phagocytosed by macrophages and the space filled with reactive astrocytes. Oligodendrocytes are relatively well preserved, but do not proliferate nor remyelinate significantly.

1.4.7. Causes of Demyelination in MS

There is still a lot of controversy over the causes of MS and the autoimmune mechanism. Venous damage is thought to be an important factor in the pathogenesis of MS, resulting from inflammatory (including immune) agents coming from the blood (Adams, 1989). It is generally thought that a viral infection is the underlying trigger for the autoimmune reaction though, as already discussed, this may not be a specific virus.

Identification of the immunological target has always been a problem. Use of phage displayed random peptide libraries to identify phagotopes binding to IgG in the CSF of MS patients has shown that CSF antibodies from different MS patients display different specificities. When serum was screened, anti-phagotope antibodies were equally frequent in the serum of healthy individuals and MS patients (Cortese et al, 1998). In another study using a phage peptide library, CSF from 5 out of 14 MS patients and 1 out of 14 controls selected an amino acid sequence motif that is found in the Epstein-Barr virus nuclear antigen (EBNA-1) and a heat shock protein alphaB crystallin (Rand et al, 1998). Nearly all MS patients' sera were positive for EBV antigen, with raised antibody to

EBNA in their CSF. Using antigen-specific immunoblotting, the antibodies to the EBNA-1 motif in CSF corresponded to a subset of oligoclonal bands.

In another study, Kaiser et al (1997) screened 185 patients with MS, 130 patients with other inflammatory diseases of the CNS (OID) and 50 patients with spinal disc syndrome (controls). IgG antibodies in serum to CNS proteins were found in 10% of MS samples, 22% of OID and 8% of controls. In the CSF, IgG antibodies to CNS proteins were found in 6% of MS samples, 28% of OID and 0% of controls. Although as much as 97% of MS patients showed oligoclonal IgG bands in the CSF, specificity of the bands for CNS proteins was demonstrated in only 1% of these.

A number of CNS constituents have been proposed as the immunological target. Myelin basic protein (MBP) has been implicated, since high levels of anti-MBP have been found in the CSF of MS patients (Warren and Catz, 1993) and a region of peptide of MBP has been found to be associated with HLA-DR2 (Zang et al, 1998). But although injection of myelin from one animal into the brain of another of the same species causes experimental allergic encephalomyelitis (EAE) - an inflammatory disorder similar to MS - it produces only minimal demyelination. In contrast, strong demyelination is observed with injection of whole brain extracts, suggesting that the demyelination agent is not a localized component of myelin, (i.e. MBP, PLP or MAG), or that several auto-antigens are involved (Zanetta, 1996).

Another putative target for the demyelinating autoimmune response is MOG, although anti-MOG antibodies are not especially found in the CSF or blood of MS patients. Anti-MOG monoclonal antibodies cause demyelination in cultures in the presence of complement. (Xiao et al, 1991). Furthermore it has been shown that antibodies to MOG and galactocerebroside (GalC) induce MBP degradation and destabilisation of myelin, mediated by neutral proteases (Menon et al, 1997). Another

study showed that the autoimmune response towards MOG predominates over that towards MBP, and identified reactivity directed to three epitopes in MOG (Kerlero de Rosbo et al, 1997).

CSL has been proposed as an immunological target in MS. It is found not only in oligodendrocytes and Schwann cells but also in neurones and astrocytes, at the surface of cilia and in the tight junctions of ependymal cells lining cerebral ventricles (Perraud et al, 1988). Moreover CSL is not specific for nervous tissue but is widely distributed in the mammalian body (Zanetta et al, 1990). Anti-CSL antibodies have been shown to cause demyelination *in vivo* and large amounts of such antibodies have been found in the blood and CSF of MS patients. It is conjectured that CSL antibodies, produced in the blood against systemic CSL, affect the ependymal barrier, either alone or with a complement component, and allow macrophages and lymphocytes to infiltrate the brain and produce multiple sclerosis plaques (Zanetta et al, 1990). The triggering of the autoimmune disease may not be directed at CNS tissue, but other tissues – for example the PNS, liver, kidney or intestine. Or it is possible that CSL itself may not be the trigger, but an exogenous mannose specific lectin of bacteria, protozoa or plant origin. Measurement of CSF anti-CSL antibodies has been proposed as a diagnostic test for MS, with 85% specificity and 93.5% sensitivity (Zanetta et al, 1994).

Another line of inquiry into the cause of MS is the effect of free radicals on CNS tissue. Production of the free radical nitric oxide (.NO), which can be induced by interferon-γ in an inflammatory response, has been correlated with clinical signs of EAE in rats (Hooper et al, 1998). It is unproven yet whether .NO, which has a short half-life *in vivo*, exerts a toxic effect on the CNS cells directly or reacts with superoxide ($.O_2$), in an inflammatory response, to form the more toxic peroxynitrite ($ONOO^-$). This can cause lipid peroxidation and tyrosine nitration, and plaques in the brains of MS patients have

been shown to contain nitrotyrosine residues. Uric acid, a strong deactivator of peroxynitrite, was found to diminish clinical signs of a disease resembling EAE in mice. The findings that patients with MS have significantly lower levels of serum uric acid than controls, and that gout is rarely found in combination with MS, suggest that hyperuricaemia may protect against MS.

Finally, there is the REGA model of autoimmunity discussed in Section 3.5. This takes into account the possibility that any or all of the components of myelin, or degradation products of them, may be autoimmune targets and could explain the role of oligoclonal IgG. Certainly no single component as target exactly fits the picture presented by MS, and with many aspects of the disease unclear, a broad view of the disease process should be taken.

1.4.8. Remyelination and Treatment

Provided that the axon is intact, CNS myelin may be repaired by oligodendroglia, although repair and remyelination in MS is controversial (Adams, 1989). Thinly myelinated areas, often referred to as "shadow plaques", are thought to be areas of remyelination (Ludwin, 1987). Repair of myelin in the CNS is slower than in the PNS, and is thought to be impeded by growth of glial fibres from astrocytes that form in MS plaques. Clinical recovery is not necessarily an index of remyelination, as nerve conduction can improve after the formation of only a few myelin lamellae.

There is no specific treatment for MS. Steroids, such as high dose intravenously administered methylprednisolone, may be given for their anti-inflammatory effect (Frequin et al, 1992; Wandiger et al, 1998). A recent therapy - repeated doses of intravenous immunoglobulin G, thought to suppress T cell reactivity and promote remyelination - has had mixed success (Francis et al, 1999; Pashov et al, 1998; Lisak, 1998). Currently on trial is interferon-β, which induces disease-limiting cytokines.

Oestriol, suggested to be the cause of the amelioration of MS during late pregnancy, has been shown to reduce the severity of EAE in mice (Kim et al, 1999), and may provide the basis of a novel therapy for MS. Another therapeutic approach, the complete renewal of the immune system by removing stem cells by plasmaphoresis, has given positive results so far.

1.4.9. Diagnosis and Disease markers

Diagnosis of MS is made on clinical signs including evoked potentials, with additional information from brain scans and laboratory investigations of CSF. MRI scanning, described earlier, is superior to computerised tomography (CT). The laboratory tests include measurement of CSF total protein and IgG, usually expressed as a percentage of the total protein, and cell count: lymphocytes are typically slightly increased. The IgG index, calculated from IgG and albumin concentrations in CSF and serum, purports to give an indication of the integrity of the blood-brain barrier (Andersson et al, 1994). The presence of oligoclonal IgG in CSF is highly indicative of MS, although it is also found in other diseases of the CNS. Detection is usually by isoelectric focusing of paired samples of CSF and serum from the same patient with detection rates of about 95%. (Cowdrey et al, 1990). More will be said about oligoclonal IgG in the next section.One of the problems with MS, both with the treatment of an individual and with clinical trial of a treatment, is the lack of a suitable and specific marker to monitor the activity and course of the disease. The reference marker is the Expanded Disability Status Scale (EDSS) based on several units of disability (Kurtzke, 1983). However it is not an ordinate scale, one point in the scale is not a unit of disability and there is poor understanding of its relationship to pathogenesis. The MRI scan is expensive and moreover its interpretation does not agree well with clinical situation and pathology. There are a number of possible markers in CSF, for instance anti-CSL antibodies (Zanetta et al, 1994), anti-MBP, immunoglobulins or C3/C4 complement factors. However none

is satisfactory in all situations, and also would entail performing undesired lumbar punctures. Various blood markers have been suggested, such as cytokines, but there is poor correlation with disease activity. Urine markers include MBP derivatives, neopterin, and free light chains, but again there is poor correlation with disease.

1.4.10. Oligoclonal IgG

As already mentioned, the CSF of MS patients on isoelectric focusing, typically displays a distinctive pattern of banding of oligoclonal IgG (Figure 1.15). These bands occur in conjunction with the diffuse polyclonal IgG, also found in normal CSF. They are thought to represent the restricted clones that can be produced from the relatively small

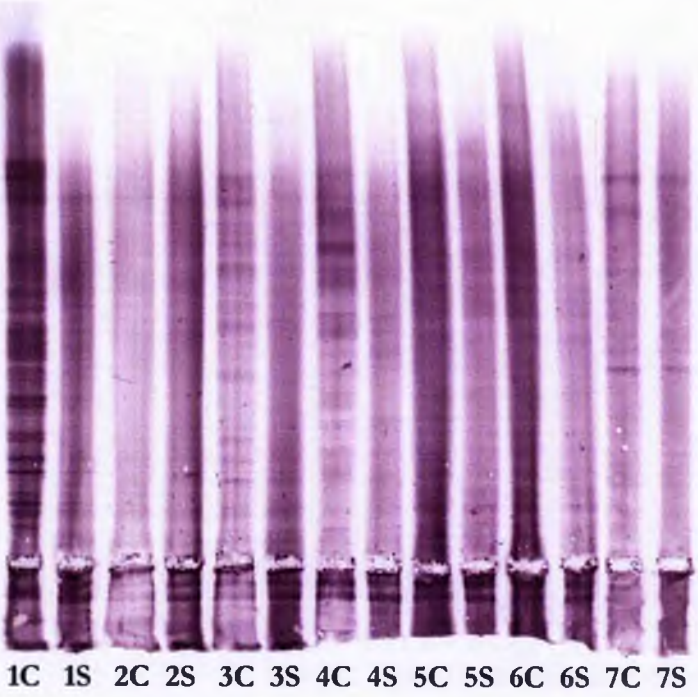


Figure 1.15. PVDF blot of routine IEF of paired samples of CSF (C) and serum (S) run on acrylamide gel with immobilized 7 to 10 pH gradient. Detection was with sheep anti-human IgG/anti-sheep labelled with alkaline phosphatase/5-bromo-4-chloroindoxyl phosphate and nitroblue tetrazolium. Pairs 1, 3 and 4 are positive for oligoclonal IgG; pairs 2, 5 and 6 are negative; pair 7 shows non-intrathecal IgG (similar bands in CSF and serum). Courtesy of Dr G. Cowdrey, Biochemistry Dept., Princess Royal Hospital.

number of CSF lymphocytes in MS and may even be specific for each plaque (Adams, 1989).

Oligoclonal IgG is not known to play any part in MS disease *per se*. It is detected in about 95% of cases of MS in Western patients, but in Japanese MS patients only a 56% positive rate was found (Fukuzawa et al, 1998), with the negative oligoclonal bands in MS associated with the HLA-DR4 antigen. Although some of the CSF oligoclonal IgG contains antiviral antibodies (Rand et al, 1998; Reiber et al, 1998; Frederiksen and Sindic, 1998), it is not certain to which antigens or epitopes the bulk of these antibodies are directed. It has been suggested that oligoclonal immunoglobulins might assist in the elimination of specific antigens (Opdenakker and Damme, 1994). Thus in the REGA model of autoimmunity, peptide fragments from myelin might be combining with the immunoglobulin. These would include fragments of MBP, PLP, MAG and MOG (Figure 1.11).

One characteristic of the oligoclonal bands is their high pI compared to the broad pI range of polyclonal IgG. Hence a pH gradient from about 6 to 12 is used with IEF to gain better resolution from polyclonal IgG. The high pI could be for several reasons: (1) There could be simply a difference in amino acids, with more basic amino acids occurring in oligoclonal IgG. (2) There could be a difference in the glycosylation. Negatively charged sialic acid is the only N-linked glycan that can confer a charge difference to the IgG molecule. So it has been suggested that there is a deficiency of sialic acid in oligoclonal IgG. (3) Oligoclonal IgG may be combining with peptides or fragments of peptides which are basic in nature, and confer a positive charge to the antigen-antibody complex. This fits with the REGA system of autoimmunity, which proposes the release of immunogenic peptides by proteases. Degradation products of MBP would be positively charged. One of the objectives of this project will be to investigate these possibilities.

1.5. Aims of the Project

MS is a debilitating disease whose cause is not well understood and for which there is no satisfactory treatment. Any research that would help in the understanding of the mechanism of the disease would be justified. The two major questions concerning MS are: -

- What is the cause - i.e. what is the aetiological agent? For example is there a virus, or range of viruses, triggering the disease?
- What is the mechanism of the autoimmune reaction? What is the autoantigen, or range of autoantigens?

This project has not attempted to answer directly either of these questions. However IgG is very involved in the immunology of MS, and recent research (Rudd et al, 1998; Dubois et al, 1999) shows that glycosylation plays a major role in the immune system. Therefore, the finding of any glycosylation changes that occur in IgG with MS might induce a better understanding of the role of that molecule in the disease. For instance, with rheumatoid arthritis (RA), the finding that agalactosyl IgG is prevalent has opened up research into that disease and increased greatly our understanding of its pathogenesis.

Rather than starting with a question to be answered, or a direct line of investigation, realization of problems concerning MS has unfolded as the project has progressed, and this has shaped the order of the experimental work. Experimental work is necessarily bound by the constraints of available equipment, expertise and samples, as will be seen. The problems addressed in this project can be arranged as follows: -

1) Does glycosylation of CSF IgG change in MS compared with normal? This would be in line with other autoimmune diseases, notably rheumatoid arthritis.

2) If there are changes, what are they in terms of the glycans or the monosaccharides of IgG? Incidental to this would be the determination of the glycosylation pattern of IgG in CSF from normal patients and those with MS. This has not yet been published. Is the glycosylation pattern of IgG in CSF the same as that in serum?

3) Do the changes (if any) contribute to the pathogenesis of MS and, if so, how? For example would lack of a particular glycan or residue of a glycan affect the structure and function of IgG? Could it thus be either a symptom or a cause of the disease? There would probably not be enough time to address this problem experimentally in this project, but conjectures could be made from the studies of glycosylation.

4) What is the character of the oligoclonal IgG bands? Could their alkaline isoelectric points be due to changes in IgG's amino acids, or to changes in the saccharides (such as a lack of sialic acid), or to IgG's complexing with peptide fragments such as degradation products of myelin?

Chapter Two

Techniques in Glycoanalysis

2.1. Methods of Glycoconjugate Analysis

This section will look at the methods available for the analysis of carbohydrates in glycoconjugates. A more detailed study of methods used in this project will be given in section 2.2.

The analysis of glycoconjugates ranges from the identification of monosaccharides to characterization of the oligosaccharide chains with determination of monosaccharide sequences and linkages (Hounsell, 1997). Analysis of the oligosaccharides may be performed while they are *in situ* on the protein, or after they are released and separated.

2.1.1. Chemical Methods

2.1.1.1. Periodation

One of the oldest methods for analyzing carbohydrates involves the use of periodic acid, which has particular relevance to the analysis of oligosaccharides as will be seen. Malaprade in 1928, in his chemical estimation of glycols, showed that the oxidation of substances with vicinal hydroxyl groups could be shown by the equation

$$\begin{array}{l} CH_2OH \\ | \\ (CH_2OH)_n + (n+1)HIO_4 \\ | \\ CH_2OH \end{array} \rightarrow 2HCHO + nHCOOH + H_2O + (n+1)\,HIO_3$$

whereby the secondary hydroxyl groups yield formic acid and the primary alcoholic groups formaldehyde (Marshall and Neuberger, 1972). Glucose is oxidized thus by periodic acid, the anomeric carbon yielding formic acid. Quantitation is achieved by

measurement of periodate, formaldehyde or formic acid (Furth, 1988). This reaction is also the first reaction in the histological stain used to detect carbohydrate in tissue: the periodate, under the correct conditions does not further oxidize the aldehydes, and these combine with Schiff's reagent, fuchsin-sulphurous acid, to give a substituted dye (Pearse, 1968). The periodate oxidation reaction has been much studied, and some points worth noting are (Marshall and Neuberger, 1972): 1) secondary reactions occur as well as the oxidation of glycols, depending on reaction conditions; 2) low temperature, i.e. between 0 and 4°C, produces less secondary reaction; 3) optimal periodic acid concentration is between 0.005 and 0.1 M; 4) optimum pH is between 3 and 4.5; 5) light decomposes metaperiodate solutions, producing ozone which accelerates the periodate oxidation of non-glycol compounds; 6) amino acids may interfere in the reaction by being oxidized by periodate.

Various uses for periodate oxidation have been found in glycoanalysis. It has been successfully used in this project to deglycosylate antibody. It can be used to sequentially release monosaccharides from the non-reducing end of a glycan chain, with intermediate mild hydrolysis steps (Hounsell, 1997). In combination with methylation, it has been used for oligosaccharide analysis with mass spectrometry. (Angel and Nilsson, 1988). Reaction conditions can be adjusted to specifically cleave the glycerol side chain at C6 of sialic acids, or to oxidize the reduced end of oligosaccharide alditols, enabling labeling of sialylated oligosaccharides or reductively aminating and sequencing *O*-linked chains (Stoll and Hounsell, 1988). Periodate oxidized carbohydrates of *N*- and *O*-linked glycans will react with biotin-hydrazide. The incorporated biotin can then be bound by streptavidin-alkaline phosphatase which will act on a substrate to produce a colour change. This is the basis of at least two kits on the market for detection of glycosylation.

2.1.1.2. Hydrazinolysis

Anhydrous hydrazine, since its introduction in the 1960s, has often been used to release both *N*- and *O*-linked chains from peptides (Yosizawa et al, 1966). Under controlled conditions, it will give a high yield (>85%) of intact *N*- and *O*-linked glycans in true molar proportions (Patel et al, 1993). The glycans are released with a free reducing terminus, rendering them available for labeling. Reaction conditions can be varied to selectively release only *O*-linked oligosaccharides (60°C for 5 h) or both *N*- and *O*-linked (95°C for 4 h). Excess hydrazine is removed by evaporation at 25°C. Primary amino groups generated during the reaction are removed by addition of excess acetic anhydride in saturated sodium carbonate. Sodium ions and peptide material can be removed by column chromatography.

This method has been automated by Oxford GlycoSystems for the release of both *N*- and *O*-glycans, or only *O*-glycans, using the GlycoPrep 1000. The intact glycans produced are ready for labeling and analysis.

2.1.1.3. Other Chemical Methods.

Methylation has been a useful technique in structural carbohydrate chemistry for some time, often used in conjunction with hydrazinolysis or periodation to release the sugars (Marshall and Neuberger, 1972). β-elimination under mild alkaline conditions has been used to release *O*-linked oligosaccharides (Fukuda, 1989). Very strong alkali (Lee and Scocca, 1972) or lithium aluminium borohydride (Likosherstov et al, 1990) have been used for both *N*- and *O*-linked glycans. These reagents cause extensive degradation of alkali-labile glycosidic linkages unless performed in the presence of excess reducing agent, which leads to the recovery of oligosaccharides as the reduced alditol derivatives.

2.1.2. Biochemical Reagents

Anti-carbohydrate antibodies, lectins and carbohydrate-processing enzymes may recognize not only monosaccharide types, but also their linkage positions, anomeric

configurations and sequence (Hounsell, 1997). Monoclonal antibodies are now available and with plant, marine and microbial lectins, a wide range of specificities is offered. Mammalian carbohydrate-binding proteins, such as selectins, collectins, galectins and sialoadhesins, are becoming available as recombinant proteins, and tend to recognize more complex conformational motifs (tri- to octo-saccharide sequences) than enzymes or lectins. Glycosyltransferases and glycosidases are also now available from recombinant technology.

2.1.2.1. Enzymes

a. Proteases and Endoglycosidases

Several methods of oligosaccharide analysis, such as Mass Spectrometry (MS) High Performance Liquid Chromatography (HPLC), Nuclear Magnetic Resonance (NMR) and Capillary Zone Electrophoresis (CZE) require that glycans be released from peptides. This can be accomplished by hydrazinolysis, as described above, or by using one of a variety of endoglycosidases or proteases (Table 2.1). These are derived from bacterial or recombinant sources, and can be obtained from several suppliers of glycoanalysis reagents. Purity of the enzyme and absence of other proteases is important. Denaturation of the glycoprotein is usually recommended to achieve complete cleavage of glycans with minimal amounts of enzyme.

b. Sequential Exoglycosidase Digestion

Exoglycosidases cleave non-reducing terminal monosaccharides from glycan chains, and can be used step-by-step to determine the sequence of monosaccharides. This is usually performed after release of the glycan from the peptide by hydrazinolysis or endoglycosidase digestion. After each digestion, the remaining oligosaccharides, appropriately labeled, can be analyzed by polyacrylamide gel electrophoresis (PAGE) or

Table 2.1. Some examples of enzymes used to release oligosaccharides (Hounsell, 1997)

Enzyme	**Specificity** (the arrow signifies the cleavage point)
Endoglycosidase H (Endo-H)	$(Man)_nGlcNAc\beta1\text{-}4GlcNAc\beta1\text{-}Asn$ (protein)
Peptide *N*-glycanase (-glycosidase) F (PNG-ase F)	NeuAc ±Fuc ±Gal $(GlcNAc)_n$ $(Man)_3$ $GlcNAc\beta1\text{-}4$ $[\pm Fuc\alpha1\text{-}6]GlcNAc\beta1\text{-}Asn$ (protein)
Endo-α-*N*-acetylgalactosaminidase	$Gal\beta1\text{-}3GalNAc\alpha1\text{-}Ser/Thr$
Endo-β-galactosidase	$[\text{-}3Gal\beta1\text{-}4GlcNAc\beta1\text{-}]_n \pm 6SO_4$

HPLC. For example, in the fluorophore-assisted carbohydrate electrophoresis (FACE) system, produced by Glyko Inc., aliquots of labeled oligosaccharide sample are incubated with different combinations of mixtures of up to five different glycosidases before running on PAGE. Alternatively, the released monosaccharides may be analyzed by high pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), as in the Dionex Ltd. system (Weitzhandler et al, 1994).

With computerized data analysis, an array of enzymes can be used to determine the oligosaccharide profile of a glycoconjugate. This is the basis of the Reagent Analysis Array Method (RAAM) analyzer marketed by Oxford GlycoSystems. *N*- and *O*-glycans are automatically released by hydrazinolysis and labeled with the fluorophore 2-aminobenzamide (2-AB) or with tritium. Automated sequential digestion is followed by chromatography of oligosaccharides and then data analysis.

2.1.2.2. Lectins

The biological properties of some lectins have been described in Chapter One. Lectins can be found in many organisms and are involved in numerous cellular processes that depend on specific recognition and binding of complex carbohydrates. As such, many lectins are available as analytical tools, and offer a wide variety of carbohydrate

targets. A general overview of lectin methods will be shown here, with a more detailed description of lectins relevant to this project given in the next section.

a. Classification

Carbohydrate-binding proteins can be divided into two major groups (Rini, 1995). Group I consists of proteins and enzymes, such as bacterial periplasmic transport proteins, which have a buried binding site and engulf the ligand fully upon binding. Group II has a shallow binding site, mostly in the form of a depression on the protein surface. This allows for a 'loose' fit in binding, and binding to several ligands may be possible, with a range of avidity. In this group are the legume lectins, c-type lectins and galectins, as well as other plant lectins, viral proteins, toxins, anti-carbohydrate antibodies and pentraxins (Weis and Drickamer, 1996).

b. Sources

Lectins derived from plants are usually multimeric and soluble proteins (some lectins are themselves glycoproteins) with multiple binding sites for carbohydrates (Cummings, 1997). The multivalent nature of plant lectins allows them to agglutinate cells to which they bind, and a number in plants were identified historically as agglutinins.

Many lectins have been characterized from leguminous plants, and often have shared primary structures. These include pea lectin (PSA) from the common garden pea (*Pisum sativum*), concanavalin A (Con A) from the jack bean (*Canavalia ensiformis*), *Ricinius communis* agglutinin (RCA) from the castor bean, and *Phaseolus vulgaris* agglutinin (PHA) from the red kidney bean. The non-leguminous plant lectins comprise a large list. For example lectins from the nightshade family include tomato lectin, *Lycopersicon esculentum* (LEL), potato lectin, *Solanum tuberosum* (STL), and jimsonweed (*Datura stramonium*) lectin (DSL). These are closely related in term of structure and carbohydrate-binding specificity and have no similarity to legume lectins in their primary structure.

Animal lectins have not been as available as those from plants, largely due to the higher cost of their preparation, but more are appearing on the market. They include the eel lectin (*Anguilla anguilla*), snail lectin (*Helix pomatia*) and horseshoe crab lectin (*Limulus polyphemus*).

An extensive range of affinity-purified lectins is available from several different companies. Many are available as conjugates of various reagents such as biotin, peroxidase, digoxigenin, fluorescein isothiocyanate or other fluorescent tag, as well as alcohol dehydrogenase, ferritin and colloidal gold, or they may be immobilized on agarose, acrylic beads or Evans Blue.

c. Binding

Carbohydrates interact with lectins through hydrogen bonds, metal coordination, van der Waals forces and other hydrophobic interactions (Elgavish and Shaanan, 1997). The availability of large numbers of hydroxyl groups on sugars makes them ready partners in complex networks of hydrogen bonds. Divalent cations, such as Ca^{2+} and Mn^{2+} are involved in carbohydrate recognition either indirectly by shaping the combining site, as in the legume lectins, or through the direct binding of the carbohydrate to Ca^{2+} as in the c-type lectins. For this reason, when using lectins in analysis, it is often necessary to incorporate these cations in the assay buffer. Similarly, buffers containing phosphate ions should be avoided, as these may bind the cations and render them unavailable to the lectin.

Most lectins have a primary specificity, with low affinity, for a particular monosaccharide. The selectivity towards a particular target is augmented by several orders of magnitude through multiple binding (Weis and Drickamer, 1996). This can be 'sub-site' binding where one monosaccharide binds at the primary site, and additional monosaccharides in the glycan bind at secondary 'sub-sites'. Or 'subunit' multivalency

occurs when several subunits of the same lectin bind to different extensions of a branched carbohydrate. The anomeric linkage of the sugars involved is also a factor in lectin binding. Some lectins may prefer a particular linkage on a sugar, and binding with other linkages may be reduced or absent. However some lectins will recognize and bind target sugars that have a range of linkages.

A range of substances will bind to a particular lectin with varying degrees of avidity. The more avid substances, which may not be a natural target, will then inhibit the lectin from its usual binding to other sugars. This property can help characterize lectin-carbohydrate interactions. Tables are available showing the relative inhibitory potency of various saccharides towards particular glycoproteins (Goldstein and Poretz, 1986)

d. Uses in Analysis

The agglutination properties of lectins have been studied and utilized for many years (Sumner and Howell, 1936), notably in blood grouping (Boyd and Shapleigh, 1954) and histopathology (Alroy et al, 1984). They are very much in use today as histochemical techniques, and various optimized systems are available from several companies to detect specific carbohydrates in tissue sections. Biotinylated lectins are often used which can be coupled with an avidin-enzyme complex, and reacted with a colour-producing substrate (Riley and Elhay, 1996).

In a similar way, lectins may be used to detect specific carbohydrates in dot-blot techniques (Sumar et al, 1990) or on membranes Western blotted from electrophoresis gels (Lundy and Wisdom, 1992; Ørntoft et al, 1997).

Affinity chromatography, with one or more lectins immobilized within a column, has been extensively used to differentiate the various glycoforms of particular glycoproteins (Candiano et al, 1983; Sumi et al, 1999) or glycopeptide hormones (Kobata

and Takeuchi, 1999). Elution may be with a simple buffer or with a lectin inhibitor, to enable collection of fractions containing the different glycoforms.

In lectin affinity electrophoresis, one or several lectins are incorporated into the electrophoresis gel. The separation of glycoproteins, or more exactly their relative migration distance, is dependent on the differences in dissociation constants of the lectin-glycoprotein complexes: the glycoproteins with sugar chains that have higher affinities for a lectin are retarded, revealing microheterogeneity (Taketa, 1991). Crossed affinity immunoelectrophoresis is a modification of the technique, whereby an antibody to the ligand is incorporated into the gel with the lectin in two-dimensional electrophoresis (Bøg-Hansen, 1973). Increased sensitivity can also be gained by blotting onto antibody-coated or lectin-coated membranes. These techniques have been extensively used in the characterization of the chain structures of α-fetoprotein in different liver cancers using Con A, *Lens culinaris* agglutinin (LCA), *Phaseolus vulgaris* agglutinin (E-PHA) and *Allomyrina dichotoma* lectin (Allo-A) (Taketa, 1998; Yamamoto et al, 1998; Zaninotto et al, 1996). Alkaline phosphatase isoforms have also been studied in liver and bone disease, using wheat germ lectin (WGA) (Crofton, 1992).

Lectins may be used in quantitative glycoprotein assays either as a detector system, where the target glycoprotein is attached to the solid phase (Goodarzi and Turner, 1997) or as a capture system where the lectin is attached to the solid phase, with an antibody, or some other recognition system, used for the detection of the captured glycoprotein (Parker et al, 1992). The concept may be taken a stage further, as in glycoprotein-lectin immunosorbent assay (GLIA) where an antibody attached to the solid phase captures the target glycoprotein, which can then be probed with a lectin (Kondo et al, 1995; Madiyalakan et al, 1996). There are many possible variations: assays may be competitive or non-competitive; markers may be enzyme systems, radioactive labels, chemiluminescent labels or digoxigenin, and detection may involve avidin/biotin systems (Rhodes et al,

1993). A number of lectins of varying specificities may be used to probe the glycoprotein and obtain a picture of the glycosylation pattern (Köttgen et al, 1993; Rafferty et al, 1995), which is a technique used in this project.

2.1.3. Biophysical Methods

2.1.3.1. Mass Spectrometry (MS)

Early use of MS in the glycosciences was as a discriminatory device on-line to GC (GC-MS) for the large number of possible derivatives obtained by methylation analysis (Hounsell, 1997). The problem of this - an inability to distinguish within the groups of monosaccharide - was improved with the detection methods of electron-impact MS (EIMS) and chemical ionization MS (CIMS), although these techniques are restricted by the size of the molecule that can be ionized and hence detected (<m/z 1000). Fast atom bombardment (FAB) and liquid secondary ion (LSIMS) became ionization methods of choice for oligosaccharide analysis in the 1980s (Dell, 1987), while in the 1990s, matrix-assisted laser desorption ionization (MALDI) has extended the molecular weight range to several hundred kDa, particularly with time of flight (TOF) analysis. MALDI-TOF is particularly simple and quick to use, giving molecular weight of oligosaccharides without derivitization, with easy-to interpret spectra (Whittal et al, 1995). But there are problems with this method from matrix-dependent deposition of excess internal energies, producing extensive metastable fragmentation (Burlingame, 1996). Also, electrospray ionization (ES), using a straightforward interface with microbore or capillary HPLC, is a reliable method for structural characterization of protein glycosylation by MS, at the picomole level. This approach requires that the oligosaccharides are derivatized, for instance with 2-aminoacridone (Charlwood et al, 1999), 4-aminobenzoic acid (Mo et al, 1999) or 1-aminopyrene-3,6,8-trisulphonate (Monsarrat et al, 1999).

2.1.3.2. X-ray Crystallography

This technique is valuable for studies of oligosaccharide-to-protein and oligosaccharide-to-oligosaccharide interactions. The method is viable as long as the oligosaccharides are complexed to protein (Hounsell, 1997). The 3-D models of proteins are very eye-catching, but X-ray crystallography provides more information than just graphic images.

Lectins and immunoglobulins particularly lend themselves to study by this technique. The carbohydrate-binding sites of these two types of molecules exhibit similar features of protein-carbohydrate interaction (Bundle, 1997). Antibodies possess sites generally deeper than those found in lectins, but the number of monosaccharide residues usually required to fill them - 3 to 4 hexose units - is the same in each case.

Briefly, the procedure for crystal structure determination is as follows (Bundle, 1997). Firstly, the glycoconjugate needs to be purified. This can be accomplished by affinity chromatography using antibodies, or in the case of IgG, protein A or protein G columns. A concentrated solution of the glycoconjugate is prepared and usually water is slowly evaporated. Then by one of a variety of techniques, crystal formation is initiated. A single crystal is then held in the path of a narrow beam of X-rays. Deflected beams fall on to a detector surface, often X-ray film which displays dark spots where beams impinge on the film. Or better, electronic area detectors digitize the direction and intensity of reflections. Diffraction data is then converted into electron density maps, which allows a correlation with most amino acid main chain and side chain elements of the protein, until the protein chain can be traced.

The 3-D structures of more than 1000 different proteins of various classes are collected in the Brookhaven protein data bank, but less than 100 entries deal with proteins that bind carbohydrates (Bernstein, 1977). The Cambridge structural database (Allen et al, 1991) contains structures of small organic molecules, but there are less than 1000 entries

for mono and oligosaccharides, reflecting the difficulties in crystallizing complex carbohydrates (Siebert et al, 1997).

2.1.3.3. NMR Spectroscopy

NMR spectroscopy is firmly established in the structural analysis of biomolecules in solution. It is often used in conjunction with X-ray crystallography and computerized calculations of molecular dynamics simulations for the structural analysis of oligo- and polysaccharides (Siebert et al, 1997).

One advantage of NMR is that it is a non-destructive technique, and the sample remains available for analysis by other methods. However, a large sample is initially required: normally at least 50 μg of a purified oligosaccharide to give details of the monosaccharides present and their positional and anomeric linkages (Hounsell, 1997). Structure is determined by comparison with data in the literature (Hounsell and Wright, 1990) of the chemical shifts of signals from protons in solution in D_2O, where hydroxyl ions have been replaced by DO by exchange. More sample material is needed to carry out proton NMR in H_2O, or to detect natural abundance of ^{13}C.

Information about structural conformation is based on measurement of the Nuclear Overhauser Effect (NOE). This originates from dipole-dipole interactions between molecules less than 3.5 Å apart for carbohydrates. The NOE intensity is proportional to minus the sixth power of the distance between the two protons involved. However in flexible molecules like oligosaccharides, the NOE-derived distances must be considered as time-averaged data, not necessarily leading to true conformations (Siebert et al, 1997).

2.1.4. Chromatography

Chromatography, with its many variations, may be used to study monosaccharides or glycans, or the different glycoforms of glycoconjugates.

2.1.4.1. Gel Permeation Chromatography (GPC)

Gel permeation column chromatography separates on the basis of hydrodynamic volume. This is a function of the size (molecular weight) and three-dimensional shape (tertiary structure) of a given molecule in a given solution (Yamashita et al, 1982). The method is suitable for neutral glycans, after enzymic or chemical release from peptide, and labeling with a fluorescent or a radioactive marker. It is the basis of the automated GlycoSequencer (from Oxford GlycoSystems), which gives carbohydrate composition in terms of glucose units (GU), by reference to an internal standard of partially hydrolyzed dextran. The hydrodynamic volume of an individual glycan is determined by interpolation and can be used to deduce the approximate number and types of monosaccharides it contains (Table 2.2).

2.1.4.2. Anion Exchange Chromatography (AEC)

In anion exchange chromatography, acidic glycans are eluted sequentially from a resin column by increasing the concentration of a counter ion. Separation is based on net charge, although there may be additional interaction between the uncharged body of the glycans and the resin material. The most common acidic substituents are sialic acid, phosphate and sulphate. The presence of these can be demonstrated by comparing charge profiles before and after treatment to remove them (Oxford GlycoSystems, 1994b).

Table 2.2. GU contribution of monosaccharides to hydrodynamic volume (Oxford GlycoSystems, 1994)

Monosaccharide	Hydrodynamic Volume (GU)
Galactose	1.1
Mannose	0.9
Fucose - outer arm	0.5
Fucose - core substituted α1-6	1.0
GalNAc	2.0
GlcNAc	2.0
Xylose	1.0
GlcNAc	2.0

A development of this method is high pH anion exchange chromatography (HPAEC) with electrochemical detection by pulsed amperometry (PAD) which has been used for analysis of sialic acids (Hounsell, 1994) and the carbohydrates of IgG preparations (Weitzhandler et al, 1994).

2.1.4.3. Polyacrylamide gel electrophoresis (PAGE)

There are several gel electrophoresis systems available for analysis of mono- or oligosaccharides (Hu, 1995). The carbohydrates are released from the peptide by enzymic or chemical means and then labeled with a fluorophore such as 2-aminobenzoic acid (2-AB), or in the case of Glyko's fluorophore assisted carbohydrate electrophoresis (FACE) system, with 8-aminonaphthalene-1,3,6 trisulphonic acid (ANTS). After electrophoresis the resultant fluorescent bands can be quantitated using a specialized UV imager. Alternatively, derivatization can be omitted and the bands blotted from the gel onto a nitrocellulose or PVDF membrane, and then visualized on the membrane by means of lectins or antibodies coupled, possibly via biotin and avidin, with an enzyme such as horseradish peroxidase (HRP) or alkaline phosphatase (AP). In the BioRad Immun-Blot kit, terminal non-reducing monosaccharides are oxidized with periodate, labeled with biotin, then detected with a streptavidin-alkaline phosphatase system.

With analysis of glycoproteins, sodium dodecyl sulphate (SDS) can be incorporated into polyacrylamide gel (i.e. SDS PAGE), and this will eliminate charge differences between the proteins, causing electrophoretic separation to be dependant on molecular weight (Laemmli, 1970). Hence different glycoforms of the same glycoprotein may be resolved (Weitzhandler et al, 1994). Proteins of known molecular weight can be run as internal standards to determine the molecular weights of the analyzed glycoproteins.

2.1.4.4. Isoelectric Focusing (IEF)

Isoelectric focusing is a development of PAGE in which a pH gradient is introduced into the gel to enable greater resolution on the basis of charge. The technique has not been extensively used for analysis of glycoproteins, because monosaccharide residues, apart from sialic acids, have a neutral charge. However, IEF has been used in the diagnosis of the carbohydrate-deficient glycoprotein syndromes, by studying the sialylation of glycoforms of a suitable marker protein such as transferrin in CSF (Keir et al, 1999; Schachter and Jaeken, 1999; Krasnewich et al, 1995). The technique has also been used to look at transferrin sialylation forms in screening for alcohol abuse (De Jong et al, 1995). It has been used also in studies of various glycoproteins to establish whether observed microheterogeneity is due to variability in sialic acid content or to rearrangement of amino acid composition (Larrea et al, 1995; Gianazza, 1995; Baudin et al, 1997). It is often the first separation step in glycoconjugate analysis, followed by lectin-affino chromatography (Hachulla et al, 1992); lectin-affinoblotting (Hänser et al, 1995) or HPAEC-PAD (Anderson et al, 1994).

2.1.4.5. Gas Chromatography (GC)

GC is the method of choice for the analysis of monosaccharides because of its robustness and ability to separate a large number of closely related molecules (Hounsell, 1997). The sugars need to be converted to methyl glycosides or to partially methylated alditol acetates, using methanolic hydrochloric acid, before analysis (Tomana et al, 1988; Patel et al, 1993). Quantitation of the monosaccharides, by GC or GC-MS, is best achieved after derivatization to trimethylsilyl ethers of methyl glycosides (Hounsell, 1994).

2.1.4.6. High Performance Liquid Chromatography (HPLC)

HPLC is generally a very sensitive technique adaptable to analysis of both monosaccharides and oligosaccharides. Derivatization of monosaccharides is often with 2-aminopyridine (Hounsell, 1994). Oligosaccharides are usually derivatized with

2-aminobenazamide (2AB), which labels all glycans non-selectively and allows sub-picomolar levels of sugars to be detected in their correct molar proportions (Guile et al, 1996; Routier et al, 1998). Neutral oligosaccharides can be analyzed with a reverse phase C18 column, or with a normal phase column. Acidic glycans, i.e. sialylated, may be analyzed by normal phase chromatography. Usually acetonitrile-water gradients are used for both types of chromatography (Hounsell, 1997). HPLC may be coupled with MS (Charlwood et al, 1999) or with NMR (Sidelmann et al, 1995). More will be said about the HPLC used in the project in section 2.2.5.

2.1.4.7. Capillary Electrophoresis (CE)

The high resolving power of CE makes it particularly suitable for the separation of oligosaccharides. It also has an advantage over HPLC in that it can resolve the glycoforms of intact glycoproteins (Kakehi and Honda, 1996; Hounsell, 1997).

Where charged species are being studied, such as glycosaminoglycans or sialylated glycoconjugates, no pretreatment is required for the separation of glycoforms (Iourin et al, 1996). Otherwise oligosaccharides or glycoproteins can be given a charge by complexation with metal ions or with borate (Rudd et al, 1992; Honda et al, 1991), or by derivatization with ANTS or 2-AB.

As mentioned in section 2.1.3.1, increased sensitivity can be achieved by linking the outflow from CE directly into electrospray mass spectrometry (Monsarrat et al, 1999).

2.1.5. Glycoprotein Analysis Strategy

Several companies producing reagents for glycobiology research suggest different ways of characterizing glycoprotein glycans, depending on the kits and reagents they have to offer, or various researchers suggest protocols. One strategy is given in Figure 2.1. The basic steps are essentially similar in all the strategies:

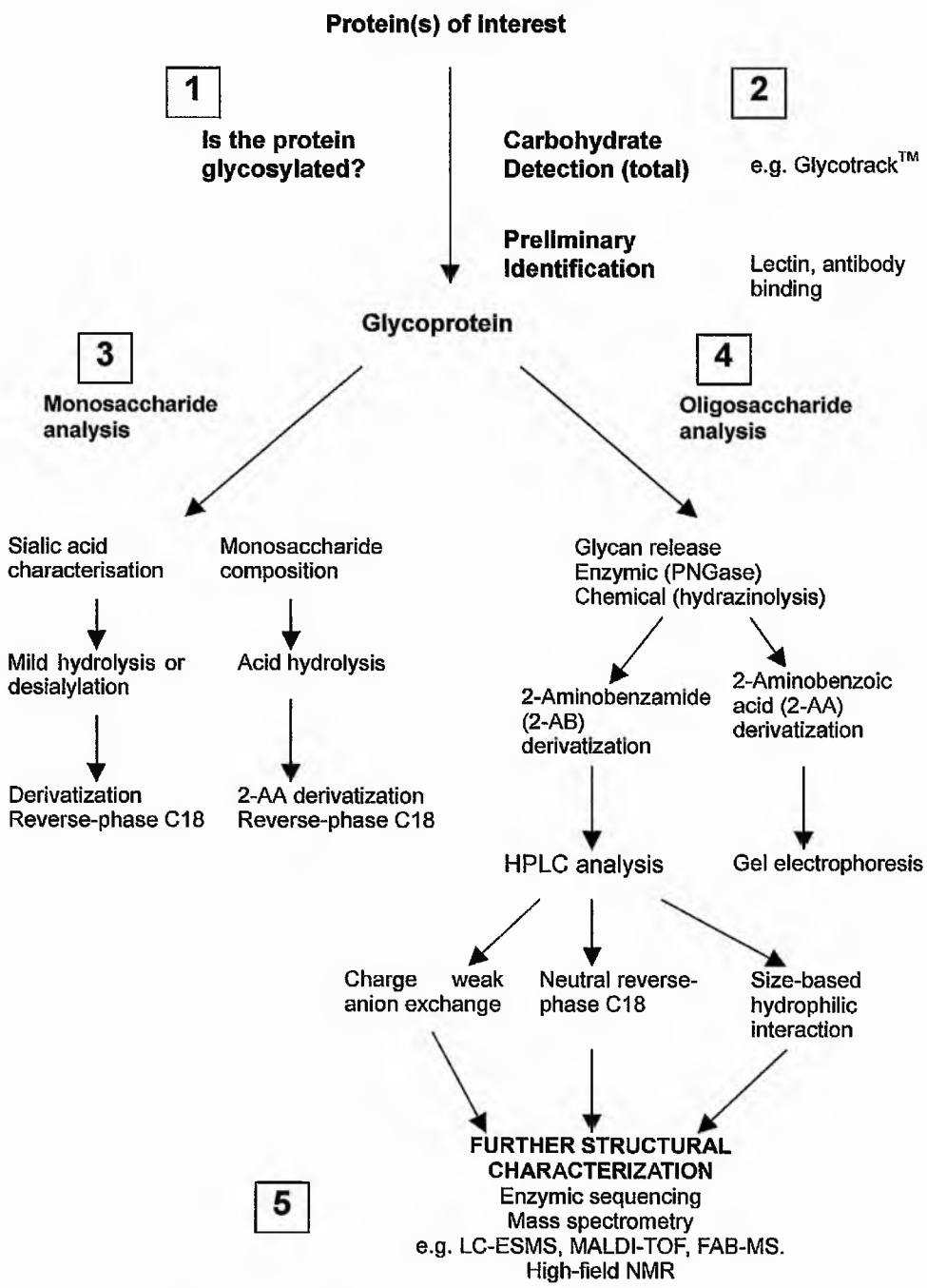


Figure 2.1. A strategy for protein glycosylation identification and analysis (Merry and Steventon, 1997). C18: carbon 18 column chromatography; GlycoTrack™: gel electrophoresis kit (Oxford GlycoSystems); LC-ESMS: liquid chromatography electrospray mass spectrometry; MALDI-TOF: matrix-associated laser desorbtion time of flight mass spectrometry; FAB-MS: Fast atom bombardment mass spectrometry; high-field NMR: generally 600 MHz or higher nuclear magnetic resonance.

1. Is the protein glycosylated? This is generally answered by means of electrophoresis with specific labeling, or membrane blotting. Or it may be a chemical assay.
2. Preliminary characterization of oligosaccharides may be achieved by lectin or antibody assays. Glycans can be released by enzymic or chemical means and identified as *O*- or *N*-glycans, for example by SDS-PAGE.
3. Monosaccharide analysis after hydrolysis can be performed by GC or GPC.
4. Oligosaccharide analysis can be performed after glycan release by chemical or enzymic means. Glycans can be labeled and analyzed by HPLC, GC, CE, PAGE, IEF, MS or NMR
5. Studies of intact glycoconjugates can be carried out by CE or MS, or by structural analysis such as X-ray crystallography.

The actual strategy used may well deviate from the classical plan depending on available sample, equipment, expertise or other factors. This is the case with this project, as will be seen in the section 2.2.

2.2. Techniques used in this project

2.2.1. Plan of experimental work

The basic strategy for this project can be divided into the five stages described in 2.1.5:

1. It is known that IgG in serum is *N*-glycosylated, and this is likely to be the case in CSF. So tests for protein glycosylation have been omitted.
2. For preliminary detection and identification of oligosaccharides in CSF IgG, a series of glycoprotein-lectin immunosorbent assays (GLIAs) using different lectins were developed and used to examine differences in glycosylation pattern between samples for MS patients and 'normal' controls. As a

necessary preliminary step, a simple immunoassay for IgG was developed, as described in Chapter Three. Chapter Four then deals with GLIA methods.

3. Monosaccharide analysis, *per se*, was not performed. The monosaccharides of IgG and their sequence are well known.
4. Oligosaccharide analysis is described in Chapter Five. Glycans were released by hydrazinolysis, labeled with 2-AB and analyzed by HPLC. A group of CSF samples from MS patients was compared with a group of 'normal' controls.
5. Further analysis of intact IgG was carried out in Chapter VI using IEF, then blotting and detecting with anti-IgG or lectin binding systems. Enzymic deglycosylation was used to characterize the carbohydrate nature of oligoclonal IgG.

2.2.2. Immunoassay

Studies of the immune system and recognition of antigen:antibody binding began in the nineteenth century, and the first quantitative immunoassay - the Precipitin Test - was developed by Heidelberger and Kendall in the 1920s (Edwards, 1985). Oudin further exploited the technique in 1948, when he demonstrated that overlayered solutions of antibody and antigen diffuse to form a visible precipitin line. Feinberg, in 1957, and Ouchterlony, in 1958, carried out the technique in a more precise way in agarose gels.

The conceptual advance made by Ekins (1960) in Britain and by Yalow and Berson (1960) in the USA was that of labeling the molecules involved in the immune reaction. Initially it was the analyte that was labeled with a radioisotope, but subsequently Miles and Hales (1968) labeled the antibody reagent. The radioisotope could be detected in minute amounts, with minimal and simple compensation for background interference. This radioimmunoassay (RIA) was seen to offer, over previous technology, increased sensitivity, specificity, simplicity, and - in that antibodies could be produced against almost

any analyte – virtually universal application. The detection limits were extended by 10^3-fold, into the pmol range (Edwards, 1985).

Antibody binds antigen by a reversible, non-covalent association:

$$Ab + Ag \underset{k_d}{\overset{k_a}{\rightleftharpoons}} Ab{:}Ag$$

where k_a is the constant for the rate of association and k_d is the dissociation constant. The Law of mass action states that the rate of a reaction is proportional to the concentration of the reactants. At equilibrium the rates are equal:

$$k_a[Ab].[Ag] = k_d[Ab{:}Ag]$$

or

$$\frac{k_a}{k_d} = \frac{[Ab{:}Ag]}{[Ab].[Ag]} = K_a$$

K_a is known as the equilibrium or affinity constant. Its values may vary from 10^6 to 10^{12} l mol^{-1}: the higher the K_a value of an antibody, the larger the proportion of antibody that will be complexed to antigen (Edwards, 1985).

RIA has played a major role in medicine and biosciences over the years, however many alternatives to radioactive labels and many variations in immunoassay design have now been introduced, as will be described in section 2.2.2.3.

2.2.2.1. Separation Methods

One of the requirements of RIA and most of other types of immunoassays is a means to separate bound label from free. Either may be measured. Yalow and Berson (1960) originally used electrophoresis. Other methods include gel filtration, adsorption, affinity chromatography, fractional precipitation, immunoprecipitation using second antibody in solution or antibody bound to a solid phase. Although the rate of the antigen:antibody reaction is reduced with solid phase antibodies, it can eliminate the need for centrifugation, reduce analysis time and enable automation (Woodhead, 1995).

One convenient and effective use of solid phase is to attach the antibody to the inside of reaction tubes, or wells of a microtitre plate. Various materials, including nylon, cellulose, silanized glass and silica, have been used for adsorption of antibodies and proteins or carbohydrate antigens (Cantero et al, 1980; Frey et al, 1993). Antibodies such as IgG can be strongly adsorbed non-covalently onto the inside of polystyrene plastic tubes, as is the case in this project, by using a coating buffer of suitable pH (Lam et al, 1993).

In many immunoassays, and especially in immunometric assays, most background noise stems from non-specific binding of the labeled antibody to the solid phase. This can be improved by suitable choice of solid phase, by using effective washing procedure (Woodhead, 1995), and by using a reagent to block areas of the solid phase unoccupied by capture antibody. The blocking reagent may be serum from the same species providing the indicator antibody (Gould, 1988), or small molecular weight proteins such as casein, bovine serum albumin (BSA), or human serum albumin, often used in combination with Tween (Lam et al, 1993). In immunoassays where glycosylation is being determined, a blocking reagent containing minimal carbohydrate is essential.

2.2.2.2. Immunoassay Design

RIA is an example of saturation analysis, where a limited amount of antibody gives rise to competition between the antigen analyte and the labeled analyte to saturate the antibody binding sites. In two-site immunometric assays (variously known as capture, sandwich or non-competitive assays), an excess of capture antibody is used, so that as the concentration of analyte increases, so too does the amount of complexed labeled antibody. Since the two antibodies recognize two separate binding sites on the antigen, greater specificity is given by these assays, and they also show lower detection limits and greater sensitivity (Jackson and Ekins, 1986; Gould, 1988). The affinity of the capture antibody is less crucial in the competitive assay since excess antibody is used. Single step

assays - where sample and labeled antibody are added together - are quicker, but two-step assays with a washing step between the addition of sample and of labeled antibody have less interference from matrix effects.

Many other assay formats are possible. With small molecules such as steroids two-site methods are inappropriate. It is difficult to label such molecules because the relative size or physical properties of the label may interfere in the immune reaction. A solution is to use labeled antibodies in a competitive reaction between the analyte and a solid phase analogue of the analyte (Wood et al, 1982).

In this project the glycoprotein-lectin immunoassay requires that the IgG in the CSF sample is immobilized by a capture antibody so that it can be probed by labeled lectins, therefore a two-site, two-step immunometric assay was adopted.

2.2.2.3. Types of Labels in Immunoassay

a. Radioisotopes

Since the nineteen-seventies alternatives to radioactive labels have been used (Woodhead 1995). Although labeling with radioisotopes uses a simple technology, and radioactive decay is unaffected by physical or chemical environment, there are a number of disadvantages. There is the safety aspect, with regulation of use and monitoring of waste disposal. Most types of radioisotopes used in analysis, such as ^{125}I have short half-lives, which limits their shelf life. There is always a step to separate bound label from free. Also the signal detected in the space of two seconds with radioactive labels such as ^{125}I is 1 to 2 orders of magnitude smaller than over the same time with enzyme or luminescent labels (Woodhead, 1995). Thus speed of detection with alternative labels is increased, maintaining comparable accuracy, and also background due to the measuring device is reduced to a minimum.

b. Enzymes

Several enzymes have found a use as labels especially in enzyme-linked immunosorbent assays (ELISAs) (Edwards, 1985). Substrates with coloured or with fluorescent endpoints can be used. Disadvantages are that an extra step is usually needed in adding the substrate of the enzyme after the Ag:Ab reaction has occurred, and also there may be varying interference of enzyme activity from different matrices.

c. Fluorescence.

Fluoroimmunoassay, often using fluorescein as a label can be a very sensitive technique. However, background fluorescence is often very high in biological samples, and also quenching may be a problem (Edwards, 1985).

d. Luminescence

Luminescence, and notably chemiluminescence, offers the sensitivity of fluorescence or enzymic detection without the problems of interferences from analytical samples, and can be as sensitive as radiolabels without the problems of reagent stability or safety aspects. It can be divided into two general types: bioluminescence, derived from a living organism, and chemiluminescence which is produced by simple chemical reactions. Both may be applied to immunoassays.

A. Bioluminescence

In bioluminescence an enzyme, such as luciferase, catalyzes the oxidation of luciferin to oxyluciferin with a concomitant flash of blue light (Edwards, 1985). This involves the conversion of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) or, in some other systems, the oxidation of reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH). The luciferase enzyme can be derived from the firefly (*Photinus pyralis*), from marine bacteria or from recombinant microorganisms (Blum and Coulet, 1994). The

different luciferases produce slightly different light flashes with the λ_{max} ranging from 560 nm for firefly to 495 nm for bacterial luciferase. An alternative enzyme is aequorin isolated from the jellyfish *Aequorea victoria.* In the presence of free Ca^{2+} this catalyzes the oxidation of luciferin with a blue flash (λ_{max}= 469 nm) which persists for about 10 s. Recombinant aequorin has also been obtained from *Escherichia coli* (Stults et al, 1992).

The disadvantages with bioluminescence immunoassays are that the enzymes are expensive, unstable and there have been batch to batch variations in quality (Rongen et al, 1994). Although bioluminescence is potentially more sensitive as a label than chemiluminescence because the photon efficiency is higher, chemiluminescent labels are more commonly used.

B. Chemiluminescence

i. The Energetics of Chemiluminescence

Chemiluminescence, as the name suggests, refers to the emission of light by a substance as a result of a chemical reaction. As such, it belongs to a class of light-emitting phenomena which include fluorescence and phosphorescence. A picture of the processes involved is provided by the Jablonski diagram (Figure 2.2). Molecules in the ground electronic state, S_0 absorb enthalpic energy which elevates them to higher electronic states, S_1 or S_2. Dissipation of this excess energy can occur in a variety of ways. Internal conversion from S_2 to S_1 can occur, as can deactivation from higher to lower vibrational levels within each singlet state. Electron spins can become unpaired and the excited molecules undergo intersystem crossing to the triplet state, T_1. If molecules lose sufficient energy to occupy the lowest vibrational level of S_1 (or T_1), they may return to ground state without light emission, and the decay takes the form of heat exchange to surrounding molecules or solvent molecules. Or, the excess energy may be dissipated in the form of light emission. Decay from the singlet states occurs in the nanosecond time scale and is referred to as fluorescence or chemiluminescence, depending on the original

source of excitation energy. Decay from triplet states occurs in the micro- to millisecond timescale and is known as phosphorescence (Pringle 1993).

Only a fraction of the energy supplied to ground state reactant molecules will finally be converted into photon emission. Thus the efficiency of a chemiluminescent reaction is reflected in the quantum yield, Q. It is generally accepted that Q is the product of three components:

$$Q = q_r \cdot q_{ex} \cdot q_{em},$$

where q_r is the fraction of reacting molecules yielding an excitable molecule, q_{ex} is the fraction of those molecules that are elevated to the excited singlet state, and q_{em} is that fraction of molecules in excited states that actually emit light. The quantum yield is often very low, typically less than 1% (Rongen et al, 1994).

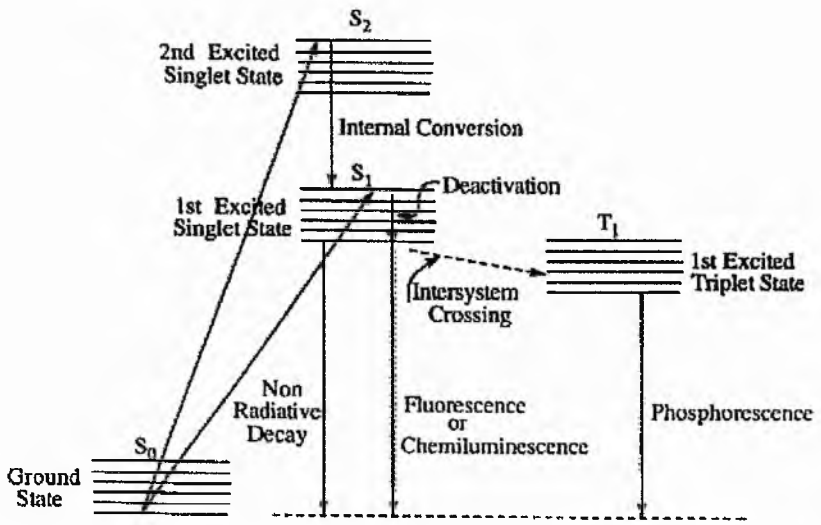


Figure 2.2. Jablonski diagram showing how the absorption of electromagnetic or chemical energy by a molecule can lead to electronically excited states and showing that the dissipation of such energy can result in luminescence emission. Maximal fluorescence/chemiluminescence emission will occur from the lowest vibrational level of the first excited singlet state, S_1, to the same vibrational level in the ground state, S_0. Intersystem crossing to the triplet state, T_1, can give rise to long-lived emission or phosphorescence (Pringle, 1993).

ii. Chemiluminescent Compounds

Most of this section will be devoted to the aminophthallic hydrazides, i.e. luminol, isoluminol and their derivatives, since they have most relevance to this project. The other compounds mentioned below are widely used, and may well have been suitable as labels in the glycoprotein-lectin immunoassays. However, isoluminol had previously been used with the luminescence analyzer available, and was adopted for the project and found to be satisfactory.

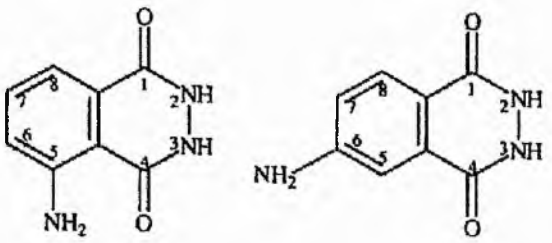
Acridinium esters are often used in luminescence immunoassays (LIA) and immunoluminometric assays (ILMA). Structurally they are similar to lucigenin. They are some one hundred times more luminescent than luminol, with Q values often above 7%. While luminol and isoluminol require an oxidant and catalyst for initiation of chemiluminescence, N-methyl acridinium carboxylic acid esters require only hydrogen peroxide. The main problem with their use is that they produce an instantaneous emission of light (Rongen et al, 1994; Pringle, 1993).

Dioxetanes are stable compounds which produce prolonged luminescence and can be used in simple immunoassay systems with a wide linear range, high sensitivity and low background. They are best used as substrates in enzyme-amplified systems: for example as meta-phenyl phosphate dioxetane with alkaline phosphatase-labeled antibody in a sandwich immunoassay (Rongen et al, 1994; Pringle, 1993).

Oxalate esters are the most efficient of all chemiluminescent compounds, and produce quantum yields of over 20%. However they are poorly soluble in water and prone to hydrolysis so that in aqueous systems the yield may be lower. They are not luminescent themselves but need a fluorescer to which they can transfer energy (Rongen et al, 1994).

The oxidations of the aminophthallic hydrazides luminol and isoluminol (Figure 2.3) are among the earliest chemiluminescent reactions to have been studied (Pringle, 1993). In aqueous solutions the quantum yield of luminol is much higher than isoluminol, however derivatization of the heterocyclic ring of luminol, or conjugation with large molecules dramatically reduces luminescence. But with isoluminol, derivatization of the amino group by alkylation is beneficial. Thus several compounds have been developed, such as the popular aminobutylethyl isoluminol (ABEI) (Figure 2.4), which can be conjugated to haptens or antibodies and still produce a high degree of luminescence (Table 2.3). Luminol can actually be used in immunoassays as a substrate, with peroxidase as a label (Arakawa et al, 1979).

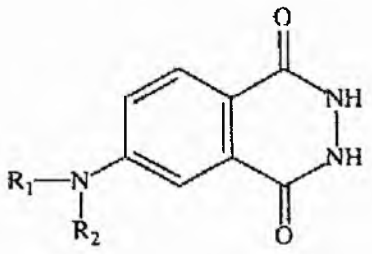
In aqueous solution the light-emitting reactions of luminol and isoluminol require



5-Aminophthalazine dione (Luminol)

6-Aminophthalazine dione (Isoluminol)

Figure 2.3. Chemical structures of the chemiluminescent cyclic hydrazides luminol and isoluminol (Pringle 1993).



R_1 = Aminobutyl; R_2 = Ethyl

Figure 2.4. Chemical structure of the isoluminol label ABEI (*N*-aminobutyl-*N*-ethylisoluminol (Pringle, 1993).

Table 2.3. Effect on chemiluminescence of amino-substitution of isoluminol (Rongen et al, 1994)

	R_1	R_2	Relative Quantum yield	Detection Limit (pMol)
Isoluminol	H-	-H	5	30
AEI	H_2N-$(CH_2)_2$-	-H	-	-
AEEI	H_2N-$(CH_2)_2$-	$-C_2H_5$	100	1
ABI	H_2N-$(CH_2)_4$-	-H	14	20
ABEI	H_2N-$(CH_2)_4$-	$-C_2H_5$	84	2
APEI	H_2N-$(CH_2)_5$-	$-C_2H_5$	-	-
AHI	H_2N-$(CH_2)_6$-	-H	17	2
AHEI	H_2N-$(CH_2)_6$-	$-C_2H_5$	44	5
AOMI	H_2N-$(CH_2)_8$-	$-CH_3$	-	-
AOEI	H_2N-$(CH_2)_8$-	$-C_2H_5$	-	-

R_1 and R_2 are defined in the structure in Figure 2.4.
Relative quantum yield is relative to luminol.
ABEI = aminobutylethylisoluminol; ABI = aminobutylisoluminol; AEEI = aminoethylethyl-isoluminol; AEI = aminoethylisoluminol; AHEI = aminohexylethylisoluminol; AHI = aminohexylisoluminol; AOEI = aminooctylethylisoluminol; AOMI = aminooctylmethyliso-luminol; APEI = aminopentylethylisoluminol.

a high pH, a strong oxidant and a catalyst or initiator. Typical oxidizing agents are hypochlorite, transition metal complex ions such as $Fe(CN)_6^{3-}$ and a variety of iron-containing compound such as porphyrins (Motsenbocker et al, 1993; Jones and Scowen, 1987), haemoglobin, peroxidases (Kricka et al, 1996) and catalase. Peroxidase may be horseradish peroxidase (HRP), or derived from bacteria (microperoxidase). Oxygen-containing species include molecular oxygen, singlet oxygen, ozone and hydrogen peroxide (Arnhold et al, 1991). Isoluminol has been used in sensitive assays to detect the release of oxygen from neutrophils (Lundqvist and Dahlgren, 1996). Horseradish peroxidase-catalyzed reactions can be enhanced by luciferin or its derivatives (Whitehead et al, 1983) or compounds such as 4-iodophenylboronic acid (Kricka et al, 1996). The amino-substituted derivatives of isoluminol, such as ABEI, may be used in competitive immunoassays, or non-competitive, sandwich-type immunometric assays (Pringle, 1993;

Rongen et al, 1994). These may be homogeneous assays, i.e. without a separation step, or heterogeneous, where bound label is separated from free.

For preparation of labels, the isoluminol derivatives are usually modified using succinic anhydride and *N*-hydroxysuccinamide to produce hydroxysuccinamide esters which can be covalently coupled to an antibody or antigen via its terminal amino groups (Van Dyke and Van Dyke, 1985). The active esters may be stored at low temperatures (i.e. -70°C for over nine months), but lyophilization is unsuitable. The conjugated steroids or proteins, however, may be stored at -20°C for at least 6 months (Messeri et al, 1989). Compounds such as ABEI can be conjugated to a variety of substances, ranging in size and complexity from steroid hormones to immunoglobulins. With steroids especially, the length of the spacer on R_1 between the steroid and the isoluminol (Table 2.3) is a factor in the luminescent properties of the conjugate (Pringle, 1993).

2.2.2.4. Immunoassays in this Project

a. Label

As mentioned earlier, isoluminol was chosen as the label in the immunoassays for the project, and as will be seen, the detector antibody was conjugated with ABEI. The trigger reagents previously used with the analyzer were also adopted: these were (1) NaOH (0.01 M) containing H_2O_2 and (2) Microperoxidase diluted in water. The chemiluminescent reaction can be summarized as follows:

$$\text{Isoluminol} \xrightarrow[\text{Microperoxidase}]{OH^- \quad H_2O_2} \text{Oxidized isoluminol} + N_2 + \text{Light.}$$

b. Assay Format

As a preliminary step to probing IgG from CSF with different lectins, a heterogeneous immunometric assay was developed for measuring CSF [IgG], then

adapted to a lectin immunometric assay (Figure 2.5). Details of the actual lectins used will be given in section 2.2.2.5.

c. Choice of Antibody

Monoclonal or polyclonal antibodies may be used in immunoassays for either the capture antibody or the detector antibody. Although monoclonal antibodies can confer

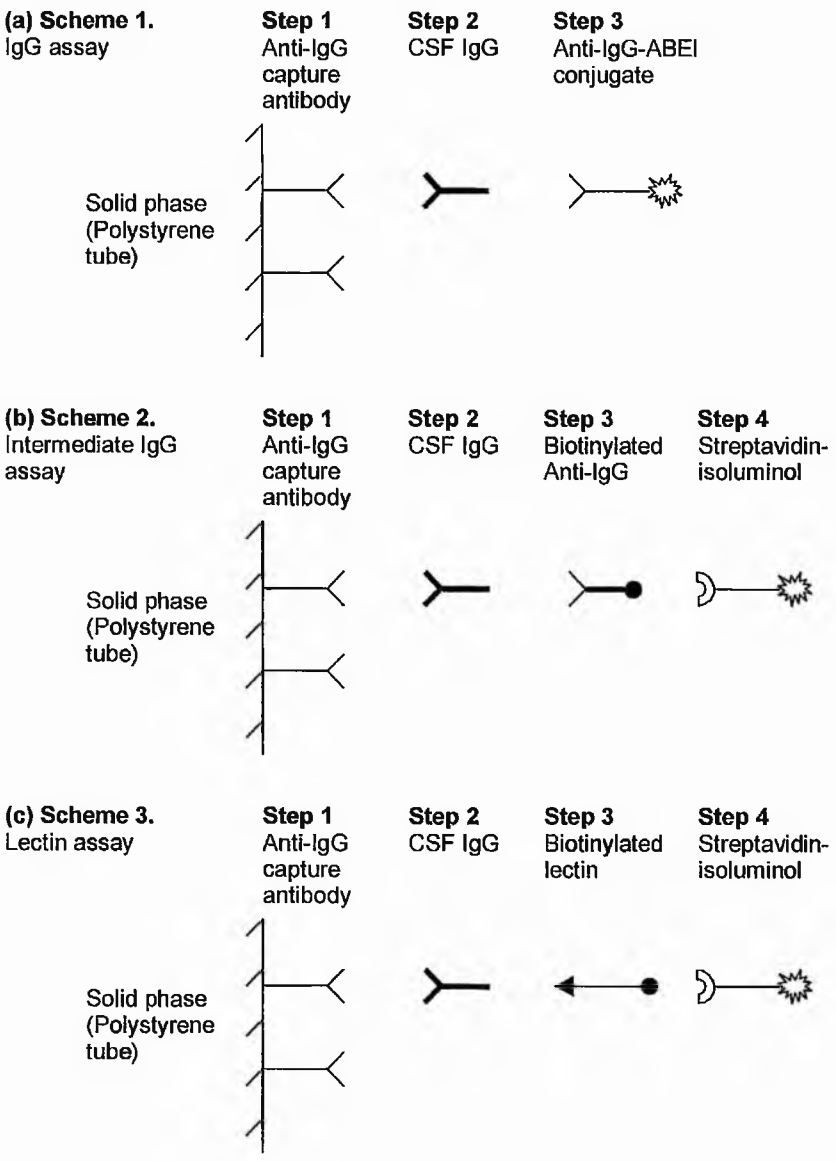


Figure 2.5. Format of immunoassays in the project. The lectin assays (Scheme 3) were developed from the IgG assay (Scheme 1) via an intermediate assay (Scheme 2). Washing was performed after each step shown.

more specificity, they have low affinity constants when compared to their polyclonal counterparts. Also monoclonal antibodies are less stable and may be prone to loss of activity with labeling or immobilization on a solid phase (Van Dyke and Van Dyke, 1989). A polyclonal antibody was chosen for this project, namely goat anti-human IgG γ-chain. This was used, for convenience, as both the capture antibody, attached to the inside of polystyrene tubes, and the detector antibody in the IgG assay, conjugated with ABEI.

d. Avidin-Biotin

The (strept)avidin-biotin interaction is well known and can be applied in several ways to enable binding of two reactants and/or multiply the binding potential of two reagents.

Biotin is a small molecule (244 Da) that can be covalently linked to several types of side chains, including those found in antibodies, lectins, enzymes and other proteins. A wide range of biotinylated compounds is commercially available. Avidin (70 kDa) is a glycoprotein of egg white and has four very high affinity sites for biotin. As it has carbohydrate residues, care must be taken in glycoconjugate analyses that non-specific binding does not occur (Danguy et al, 1997). Streptavidin, from *Streptomyces avidinii,* has a similar affinity for biotin, but a lower isoelectric point (pH 6 rather than pH 10) and has no carbohydrate (Gould, 1988).

In this project, in the immunoassays, biotin is used to link the lectins, via streptavidin, with isoluminol. Thus streptavidin-isoluminol becomes a 'universal' reagent for use with a range of different lectins. Multiple binding of biotin to the lectins leads to enhancement of the chemiluminescent signal.

Biotinylated lectins are also used in this project for detection of sugars on IgG blotted on PVDF membranes after isoelectric focusing (IEF). In this case, the label

conjugated with avidin is horseradish peroxidase (HRP), with 3,3'-diaminobenzidinetetra-hydrochloride (DAB) as chromogen.

2.2.2.5. Glycoprotein Lectin Immunoassays (GLIA)

In the 1970s lectins bound to cells had been measured or visualized cytochemically using enzymes, such as HRP, bearing carbohydrate chains (Bernhard and Avrameas, 1971). It was then postulated that lectin-antibody or lectin-antigen conjugates might be a useful tool in immuno-enzymic techniques (Guesdon and Avrameas, 1980). In general, three different approaches are possible for using lectins in immunoassays to either quantitate a glycoprotein or investigate the carbohydrate content of a glycoprotein. Firstly, the capture antibody is bound to a solid phase, sample antigen is added, followed by labeled lectin (Pekelharing et al, 1987; Rafferty et al, 1995; Tsuchiya et al, 1993; Madiyalakan et al, 1996; Casburn-Budd et al, 1992; Köttgen et al, 1993). This is the approach used in this project. Secondly, the lectin may be bound to the solid phase, sample antigen added, followed by labeled antibody (Parkinnen and Oksanen, 1989; Cullina and Greally, 1993). Thirdly, the sample analyte, after purification, is bound to the solid phase, and the lectin is added which may be biotin or digoxigenin labeled (Goodarzi and Turner, 1997) or enzyme labeled (Sumar et al, 1993; Milton and Rhodes, 1998). This method is not actually an immunoassay, and usually termed an enzyme-linked lectin assay (ELLA). In another assay, the sample antigen is complexed with fluorescein-lectin and biotinylated antibody and then streptavidin added and the whole complex captured on a biotinylated nitrocellulose membrane (Dill and Bearden, 1996). ELISA systems are most common, though other labels include ^{125}I labeled lectin (Madiyalakan et al, 1996) and europium labeled antibody (Parkinnen and Oksanen, 1989).

In lectin immunoassays of IgG, some workers have found that heat denaturation of IgG is necessary to open the tertiary structure of the molecule to expose the carbohydrate and enable access by some lectins such as *Bandeiraea simplicifolia II* (BSII) and

Ricinus communis agglutinin (RCA1) (Sumar et al, 1993). However, others have probed IgG with the same lectins (Parkkinen and Oksanen, 1989; Casburn-Budd et al, 1992), and other lectins (Parkinnen, 1989; Tsuchiya et al, 1993), without denaturation of the protein.

Purification of IgG and other analytes prior to immunoassay has been found necessary by some workers. This is usually achieved by ion-exchange chromatography (Sumar et al, 1993; Parkinnen, 1989) or affinity chromatography with protein A (Parkinnen, 1989) or protein G (Goodarzi and Turner, 1997; Tsuchiya et al, 1993). It has been found that the method of purification, i.e. ion-exchange or protein G, influences the glycosylation profile of the isolated IgG (Bond et al, 1993). Some workers have used serum in lectin assays, without prior purification of CSF IgG, relying on the capture antibody to isolate the sample IgG before probing with lectins (Casburn-Budd et al, 1992; Keusch et al, 1996; Köttgen et al, 1993).

In this project, the simplest procedure for lectin immunoassay was tried, at least initially, i.e. without prior purification or denaturation of IgG, as shown in Figure 2.5, scheme 2. Some properties of the lectins used in this project are given in Table 2.4.

2.2.3. Isoelectric Focusing

2.2.3.1. Theory

Isoelectric focusing (IEF) enables the fractionation of molecular species differing in net charge. Essentially, IEF is electrophoresis within a pH gradient: macromolecules migrate through the gradient until they reach the point where the pH of the gradient equals their pI. (The isoelectric point, or pI, of a molecule is the pH at which the molecule is in an electrically neutral form. The pI is a constant of a particular compound at specific conditions of ionic strength and temperature). Optimum resolution is obtained when concentration of the gel is lowest; therefore separation is performed in media such as polyacrylamide gels with high porosity. The method is capable of very high resolution and macromolecules differing in only 0.001 pH units can be separated (Andrews, 1995).

2.2.3.2. Gel Media

There are two ways in which a pH gradient can be introduced into a gel plate. The first to be developed was the use of carrier ampholytes. These are mixtures of low molecular weight molecules with a range of pIs, each of which in a current will migrate to the point in the gel where the pH of the media is equal to its pI (Vesteberg and Svensson, 1966). A problem with this method is that gradient drift can occur, and a way of creating an immobilized pH gradient was introduced using Immobilines™ (Bjellqvist et al, 1982). The pH gradient in the gel is usually produced by preparing two solutions of different densities and of each extreme of the pH range to be used. These are then layered and mixed using a gradient mixer: the bottom solution (usually the more acidic) is often made denser with glycerol (25% w/v). Advantages of Immobiline gels are that gradient drift cannot take place, they are less sensitive to salts in the sample, and ultraflat pH gradients can be prepared (Låås, 1998).

Although agarose gel can be used, polyacrylamide gels are more common. These can be bought pre-cast and ready to use, or pre-cast and dried, requiring rehydration before use. Broad or narrow pH ranges may be used, depending on the pI values of the analytes. The shallower the pH gradient, the further apart two different species will be. Once cast, gels need to be washed to remove residual salts (Låås, 1998).

For routine analysis of oligoclonal IgG bands a pH gradient of 7 to 10 is normally used as these bands are most prevalent in the alkaline region. However, oligoclonal IgG bands are also found as low as pH 3, so it was decided to use a broad range, i.e. pH 3 to 10, for the project. Pre-cast, dried gel strips were purchased.

2.2.3.3. Experimental Techniques

Although whole samples, or crude extracts, can be used, samples should have as low an ionic strength as possible. Too high a salt content will produce curved bands, or worse, overheating and possibly burning at the point where the salt is focused. The

Table 2.4. Characteristics of the lectins used in the project. Adapted from tables of lectins in Danguy and Gabius (1993), Vector Laboratories Inc. (1993) and Rhodes and Milton (1998).

Acronym(s)	Lectin name (common name)	Residues or sequences recognised, in order of binding preference.	Notes
BSL-II (GSL-II)	*Bandeiraea (or Griffonia) simplicifolia* (griffonia seeds)	α or βGlcNAc	Tetramer Increasing number of GlcNAc residues beyond two does not increase affinity.
Con A	*Concanavalia ensiformis* (jack bean seeds)	αMan; αGlc	Tetramer; pI = 5 Needs Ca^{2+} and Mg^{2+}
DBA	*Dolichos biflorus* (horse gram seeds)	GalNAcα1-3GalNAc; GalNAcα1-3Gal	pI = 5.5. Binds blood group A_1 cells in preference to A_2 cells
DSL (DSA)	*Datura stramonium* (thorn apple or jimson weed seeds)	$GlcNAc(\beta1\text{-}4GlcNAc)_{1\text{-}3}$ Galβ1-4GlcNAc	Sequence may be terminal or internal. Lectin binds well in acid pH: affinity decreases above pH 8.
ECL (ECA)	*Erythrina Cristagalli* (coral tree seeds)	Terminal Galβ1-4GlcNAc	Terminal sialic acid on the sequence prevents binding.
JAC	*Artocarpus integrifolia* (jackfruit)	Galβ1-3GalNAc; Galβ; NeuAc2-3Gal; NeuAc2-3GalNAc	Binding to "T-antigen" still occurs if sialylated. Has been used to purify human IgA since it will bind no other human immunoglobulin.
LEL (TL)	*Lycopersicon esculentum* (tomato fruit)	$GlcNAc(\beta1\text{-}4GlcNAc)_{1\text{-}3}$	Prefers trimers or tetramers of GlcNAc.
PNA	*Arachis hypogaea* (peanut)	Terminal Galβ1-3GalNAc	Sequence known as the "T-antigen". Sialic acid normally prevents binding. Binding is enhanced by Ca^{2+} ions.
RCA-1	*Ricinus communis* (castor bean seeds)	Galβ1-4GlcNAc; βGal; αGal	**Moderately toxic** Desialylation of glycoprotein may be required to allow access to glucose residues.
SBA	*Glycine max* (Soybean seeds)	Terminal α or βGalNAc; α or βGal	Binding can be blocked by substitutions on penultimate sugars, e.g. fucose on penultimate galactose. pI = 6.0.
SNA (EBL)	*Sambucus nigra* (elderberry bark)	NeuAcα2-6Gal; NeuAcα2-3Gal; NeuAcα2-6GalNac	Tetramer

Continued on next page

Acronym(s)	Lectin name (common name)	Residues or sequences recognised, in order of binding preference.	Notes
STL (PL)	*Solanum tuberosum* (potato)	GlcNAc(β1-4GlcNAc)$_2$β1-4GlcNAc	STL consists of 2 identical subunits, which as monomers lose their binding properties.
UEA-I	*Ulex europaeus* (furze gorse seeds)	L-Fucα	Binds blood group O cells.
VVL (VVA)	*Vicia Villosa* (hairy vetch seeds)	Terminal GalNAcα1-3Gal; GalNAcα1-6Gal; GalNAc-serine/threonine	GalNAc linked to serine or threonine is known as the "Tn-antigen".
WGA	*Triticum vulgaris* (wheat germ)	GlcNAc(β1-4GlcNAc)$_{1-2}$; β1-4GlcNAc; NeuAc	pI about 9. Can bind terminal or internal GlcNAc. Preference for dimers or trimers of GlcNAc.

Fuc = fucose; Gal = galactose; GalNAc = N-acetylgalactosamine; Glc = glucose; GlcNAc = N-acetylglucosamine; Man = mannose; NeuAc = neuraminic acid.

amount of sample to apply is a balance between the minimum intensity of the bands that may be detected and maximum intensity before bands overlap (Låås, 1998).

The sharpest bands and best resolution are obtained with maximum voltage. The limits are set by the ability of the system to control the heat produced. Manufacturers of pre-cast gels will give the preferred running conditions for their gels (Pharmacia, 1993). If the voltage needs to be reduced, running time should be extended, but this leads to reduced resolution.

To check the regularity of the pH gradient in a gel and/or identify the pI of analytes, marker proteins may be run. These can be bought as sets, ready to use, or any suitable protein, with an established pI for the same system may be used. Lists of isoelectric points for many proteins are available (Malamud and Drysdale, 1978). Table 2.4a shows the isoelectric points of some proteins commonly used as markers in IEF (Andrews, 1995).

2.2.3.4. Blotting

Blotting is the transfer of large molecules from an electrophoresis gel, or other medium, onto the surface an immobilizing membrane. The proteins adsorbed are thus fixed and, retaining their biological activities, are available for further analysis or detection techniques. Transfer methods include blotting by simple diffusion, by capillary action, using a vacuum and by electrophoresis (Westermeier, 1998). In this project, diffusion blotting is employed, simply placing the membrane on the polyacrylamide gel, followed by a glass plate and a weight. By this method, up to 90% of the proteins (Westermeier, 1998) are pressed out of the gel onto the surface of the membrane.

Polyvinylidenedifluoride (PVDF) blotting membrane (on a Teflon base) is used in this project. Compared to the commonly used nitrocellulose membrane it has a higher binding capacity and greater mechanical stability (Westermeier, 1998).

Table 2.4a. Isoelectric points and molecular weights of some proteins suitable as markers in IEF (Adapted from Andrews, 1995 and Pharmacia Biotech pI calibration kit instruction manual).

Protein	pI at 25°C	Molecular weight (kDa)
Pepsin (porcine)	2.9	33
Amyloglucosidase (*Aspergillus niger*)	3.8	97
Glucose oxidase	4.2	186
Soya bean trypsin inhibitor	4.6	20
Ovalbumin (hen's egg)	4.7	45
Serum albumin (bovine)	4.9	67
β-Lactoglobulin A (bovine milk)	5.2	36
β-Lactoglobulin B (bovine milk)	5.3	36
Carbonic anhydrase (bovine erythrocyte)	5.9	28
Conalbumin (hen's egg)	5.9	86
Carbonic anhydrase (human)	6.6	28
Haemoglobin A (bovine)	6.8	64
Myoglobin (equine), minor component	6.9	17.5
major component	7.4	17.5
Myoglobin (sperm whale) minor component	7.0	17.5
major component	7.4	17.5
Lentil lectin acidic band	8.2	-
middle band	8.5	-
basic band	8.7	-
Trypsinogen (bovine pancreas)	9.3	24.5
Chymotrypsinogen A (bovine pancreas)	9.0	23.6
Ribonuclease (bovine pancreas)	9.3	13.5
Cytochrome C (equine heart)	10.2	12.5
Lysozyme (hen's egg)	10.0	14.4

2.2.3.5. Detection

Several methods for detection of the blotted proteins are used in the project. Colloidal gold is used as a non-specific stain for proteins (and other macromolecules). For specific staining, lectins are used to identify bands containing relevant carbohydrates. The lectins are biotinylated and complex with added avidin-horseradish peroxidase (HRP). Hydrogen peroxide is then added together with the chromogen 3,3'-diaminobenzidine-tetrahydrochloride (DAB) which forms an insoluble coloured product. The avidin-biotin system, as well as enabling HRP to be used as a 'universal' reagent, aids to amplify detection. For specific detection of IgG, two systems are used: biotinylated anti-IgG with the avidin-HRP-DAB technique as for the lectins, and a system involving sheep anti-human IgG, with anti-sheep-alkaline phosphatase complex. For this latter method only, the membrane is first treated with skimmed milk to block free binding sites.

2.2.4. Protein Purification

CSF is less complicated to analyze than many other raw materials. Compared to blood, for instance, it is less viscous, and contains fewer components and normally few cells. Whole, untreated CSF is used in the project in some IEF and immunoassay methods. In other methods, IgG must be separated from other, possibly glycosylated, components of CSF, and in addition, for example for HPLC, the IgG sample must also be salt-free. Several methods of purification of IgG are used.

2.2.4.1. Salt Fractionation

Mineral salts have been used for many years for the precipitation or fractionation of proteins. Agents include magnesium sulphate, sodium sulphate and sodium chloride, but most commonly used is ammonium sulphate [$(NH_4)_2SO_4$] for precipitation of immunoglobulins (Heide and Schwick, 1973). Disadvantages are that pure immunoglobulin is not obtained without additional treatment, and the salt has to be removed by dialysis. Ethanol and acetone are also used as precipitating agents, but are more damaging to the protein. Other agents are polyethylene glycol, which is difficult to remove from fractionated protein, and Rivanol (2-ethoxy-6,9-diaminoacridine lactate). All these decrease the solubility of proteins by increasing their hydrophobicity and promoting aggregation by bringing together hydrophobic surfaces (Ersson et al, 1998).

Rivanol has been used to fractionate serum into its constituent proteins. With the correct conditions, Rivanol will precipitate nearly all serum proteins apart from IgG, transferrin, and α_1-acid glycoprotein. It has been used in analysis of transferrin glycoforms (Iourin et al, 1996), and to obtain relatively pure IgG by fractionation in combination with ammonium sulphate (Heide and Schwick, 1973).

2.2.4.2. Desalting

a. Ultrafiltration

Ultrafiltration membranes are available for separation of molecules, with cut-off limits ranging from 1 to 300 kDa. The sample needs to be fairly pure to start with to avoid clogging the pores with debris or cells (a pre-filter may be used). The method is suited for separation of salts and other small molecules from a protein fraction with a higher molecular weight, and at the same time can effect a concentration of proteins (Ersson et al, 1998).

b. Gel Filtration Chromatography

The use of de-salting columns is a rapid way of removing low molecular weight molecules. The gel used is cross-linked dextran, e.g. Sephadex, or porous polyacrylamide beads. Proteins and other high molecular weight substances (>6 kDa) elute at the void volume, whereas small molecules are retarded by the gel (Ersson et al, 1998). Ready-made, disposable columns are available.

c. Dialysis

Dialysis is a simple, but usually lengthy, method of removing salts and small molecular weight molecules from sample solutions without significant loss of macromolecules (Ersson et al, 1998). Dialysis membranes of various materials, such as cellophane, acrylic and polypropylene, are available with a range of molecular weight cut-offs. (It should be remembered that linear molecules such as DNA or RNA might pass through membranes that exclude globular proteins with the same molecular weights). Systems range from simple bags immersed in the solvent, to electrodialysis apparatus that process several samples simultaneously efficiently and rapidly (Heide and Schwick, 1973). For the project, dialysis cassettes are used that are convenient for small samples. The

sample is injected by syringe into a mini dialysis bag held in a cassette, which is then suspended under the surface of the solvent by attachment to a buoy and after dialysis sample is removed by syringe.

Salt removal depends on equilibrium being reached of distribution of contaminants between the sample phase and the solvent. The solvent is then changed a number of times with new equilibria being reached, so that contaminants in the sample phase are gradually reduced. The solvent used for simple salt removal is usually water. Trifluoracetic acid (TFA) (0.1% $^{v}/_{v}$) may be added which helps to keep macromolecules in solution.

2.2.4.3. Chromatography

There are many forms of liquid chromatography that can be applied to protein purification, based on several different separation principles. In the preparation of IgG for glycosylation analysis, ion-exchange chromatography and affinity chromatography have both been used. As has been mentioned already, human IgG prepared by ion-exchange chromatography has a different glycosylation profile to that prepared by affinity chromatography (Bond et al, 1993).

a. Ion-exchange chromatography

Ion-exchange chromatography (IEC) offers high resolution, high protein binding capacity and ease of use. Separation is based on competition between the ions of interest and other mobile ions for oppositely charged groups on the ion-exchanger. In proteins many amino acids are weak acids or bases: it is the overall charge that counts (Karlsson et al, 1998). IgG, like other proteins, has a broad range of isoelectric points, from about pH 6 to pH 10, due to its composite microheterogeneity. Some of the heteroforms will not bind to the ion-exchanger as well as others, so there is a possibility that the purified IgG will not have the same composition as the native IgG.

Nevertheless, diethylaminoethyl (DEAE) chromatography, after precipitation of immunoglobulins with ammonium sulphate, has been used by a number of workers to isolate IgG from serum samples (Parekh et al, 1985; Tomana et al, 1987; Sumar et al, 1993; Shikata et al, 1998).

b. Affinity Chromatography

Historically, affinity chromatographic techniques have exploited specific interactions found in nature. Probably the most widely used are antibody/antigen reactions, although immunoglobulin Fc receptors from bacteria have found a wide application as immobilized adsorbents. These are cell surface proteins and are classified according to their reactivity with IgG subclasses (Godfrey, 1996). Some affinity receptors relevant to IgG purification are given below.

i. Antibodies

Generally, antibody-antigen interactions have dissociation constants (K_d) in the region of 10^{-3} to 10^{-14} M at 25°C. Immunosorbent separations require that antibodies have binding affinities for their antigens, in terms of Kd values, of 10^{-6} to 10^{-10} M. Antibodies with lower K_d values (high affinities) require harsh conditions to elute the antigen, which can result in diminished recovery of active product and reduced life of the immunosorbent. Conversely, antibodies with high K_d values (low affinities) when immobilized would offer poor removal of antigen from the sample matrix. Another factor which is a drawback in the use of immunosorbents is the size of the antibody molecule which may limit accessibility of the adsorbate, while rendering immunosorbents susceptible to denaturation and/or fouling (Godfrey, 1996).

ii. Protein A.

Protein A (SpA) is a type I Fc receptor found in the cell wall of *Staphylococcus aureus* from which it may be isolated, or produced as a recombinant protein in genetically

modified *E. coli*. It is a globular protein with a single polypeptide chain and little or no carbohydrate. The molecular weight is 42 kDa, pI 4.85 to 5.15, and A_{max} is 275 nm. It is very resistant to denaturing agents such as 6 M guanidine HCl, 70%($^{v}/_{v}$) ethanol and 0.01 M HCl (Godfrey, 1996).

The SpA molecule has five homologous regions, each containing about 50 amino acids residues. Four of these (regions A, B, C and D) contain a single receptor site capable of binding to an immunoglobulin Fc region with high affinity. The fifth region (E), which is the site of attachment to the bacterial cell wall, has a low affinity for Fc binding, but will bind to the Fab region of IgG. It shares this property with protein G, and two other bacterial proteins - L and Fv (Bouvet, 1994).

The nature of the SpA-Fc interaction, as revealed by crystallography, is the formation of a major hydrophobic link between the residues of SpA's active site and the antibody's C_H2 and C_H3 domains in the Fc region (Sauer-Eriksson et al, 1995; Stone et al, 1989). There is also a minor polar link in which C_H3 residues are involved in a sulphate ion link.

iii. Protein G

Protein G (StpG) is a type III Fc receptor of group C streptococci. It has affinity for a wider ranger of IgG subclasses than SpA, especially human IgG_3, but is more expensive. Its molecular weight is 35 to 40 kDa and its A_{max} is 271 nm. It has two locations at which immunoglobulins are bound, along with four sites for albumin and cell surface binding. Like protein A, binding to immunoglobulin is in the C_H2-C_H3 region of the Fc fraction. Recombinant Protein G has been created with the albumin and cell binding sites removed, reducing non-specific binding during immunoglobulin purification (Godfrey, 1996; Sauer-Eriksson et al, 1995).

iv. Protein A/Protein G

This is a recombinant fusion protein derived from a hybrid gene comprising *S. aureus* Cowan I strain Protein A and the binding domains of *Streptococcus sp.* Lancefield group G Protein G strain. More expensive than the above, it is reputed to offer a wider range of immunoglobulin sub-class specificity, with increased adsorbent capacity (Godfrey 1996).

v. Protein H

Protein H (StpH) is a type II Fc receptor protein from group A Streptococci, which may be expressed in recombinant *E. coli.* It selectively binds human IgG and rabbit immunoglobulins only (Godfrey, 1996).

2.2.4.4. The use of protein A

In the isolation of IgG from serum, some workers have used protein A (Tsuchiya et al, 1988; Parkinnen, 1989; Newkirk et al, 1990) while others, more recently, have preferred protein G (Tsuchiya et al, 1993; Goodarzi and Turner, 1997; Wormald et al, 1997; Fleming et al 1998).

Table 2.5 shows the relative affinities of protein A and protein G for human IgG subclasses. For purification of human IgG, protein G would normally be the adsorbent of choice since it binds strongly all the IgG subclasses. However, Prosep-A, produced by Bioprocessing (no. 1 Industrial Estate, Consett, Durham, England, DH8 6TJ) was the solid phase used in the project, kindly donated by Dr P Kwasowski of Surrey University.

Table 2.5. The affinity of Protein A and Protein G for human immunoglobulins. Adapted from Godfrey, 1996).

Subclass	Affinity of SpA	Affinity of StpG
IgG_1	strong	Strong
IgG_2	strong	Strong
IgG_3	weak	Strong
IgG_4	strong	Strong
IgA_2	weak	None
IgM	weak	None

SpA: *Staphylococcus aureus* Protein A; StpG: Streptococcus Protein G.

It is said by the company that the affinity of Prosep-A for IgG_3 is maximized if the column is used below its immunoglobulin capacity. Although protein A also binds weakly human IgA and IgM, these are present in CSF in very small amounts, if at all. Table 2.6 gives the characteristics of Prosep-A and some other commercially available protein A adsorbents. Glass beads, although unstable in alkaline conditions, have otherwise excellent mechanical stability with rigid intra-particle porosity preventing compaction or fouling and increasing longevity (Godfrey et al, 1993). In carbohydrate analysis, glass is preferred to agarose from which leakage of sugars into the IgG eluate may occur, producing false readings.

High concentrations of inorganic salt (e.g. NaCl > 1 M) may be added to the adsorption buffer to promote binding of the weaker-binding subclasses (i.e. IgG_3). However this also increases the level of contaminating proteins (e.g. albumin, transferrin) in the eluate.

Table 2.6. Characteristics of some commercial SpA solid phases (adapted from Godfrey et al, 1993)

Solid phase	Matrix composition	SpA	Capacity monoclonal Ab (mg)
ACL	4% cross-linked agarose	'Unknown'	5.3
Bio-Rad	'Polymer'	Native	3.5
NYGene	Cellulose	Recombinant	5.1
Pharmacia fast flow	4% cross-linked agarose	Native	5.0
Prosep A 'high capacity'	Controlled pore glass	Native	15.0
Repligen	6% cross-linked agarose	Recombinant	2.2
Sepharose CL-4B	4% cross-linked agarose	Native	4.3
Sigma fast flow	4% cross-linked agarose	Native	5.6

The efficiency of the column may be increased by adding glycine (up to 2 M) to the adsorption and wash buffers (Godfrey, 1996).

Elution of immunoglobulin from the adsorbent is usually achieved by causing a reversible shift in the tertiary structure of the immunoglobulin such that the receptor-antibody binding is disturbed. Some examples of non-specific desorption buffers are given in Table 2.7.

Table 2.7. Examples of non-specific eluants (adapted from Godfrey, 1996; Ruoslahti, 1976).

Technique	Elution conditions
Altering solvent pH	Acid range (pH 1.5 - 4): proprionic acid (0.01-0.1 M); glycine-HCl buffer (0.01-0.1 M, pH 1.5-3) Alkaline conditions (pH 11-13.5): KCl (0.055 M); NaOH (0.145 M).
Reversible protein denaturation	Urea (<8 M). Guanidine hydrochloride (<6 M).
Polarity-reducing agents	Dioxane (<10% v/v). Ethylene glycol (<50% v/v)
Chaotropic agents	Anions (from best to worst): CCl_3COO^- > SCN^- > CF_3COO^- > ClO_4^- > I^- > ClO_4^- > NO_3^- > Br^- > Cl^- >CH_3COO^- > SO_4^{2-} >PO_4^{2-}. Cations (from best to worst): NH_4^+ > Rb^+ > K^+ > Na^+ > Cs^+ > Li^+ > Mg^{2+} > Ca^{2+} > Ba^{2+}. These salts are used at concentrations of 1.5-8 M.

Harsh denaturing conditions can irreversibly denature antibodies, so contact time with eluants should be minimized by using small elution volumes or high flow rates (Godfrey, 1996). Proteins are easily split at peptide bonds involving the aspartyl residues, and sialic acid is cleaved off under relatively mild conditions (Ruoslahti, 1976).

2.2.5. HPLC

HPLC as a method for oligosaccharide analysis has already been mentioned in Section 2.1.4.6. The HPLC assays used in the project, with preliminary *N*-glycan cleavage by hydrazinolysis and labeling with 2-AB, are methods set up at the Oxford Glycobiology Institute. Preparation of the samples, at the Princess Royal Hospital, consisted of

isolation of IgG from CSF by Protein A chromatography followed by dialysis to remove salts and other low molecular weight material.

Normal phase HPLC is an established technique for separating mixtures of oligosaccharides (Guile et al, 1996). The earliest methods used columns with diethylaminoethyl functional groups to separate neutral and acidic oligosaccharides. More recently, columns with imide and amide functional groups have been used (Townsend et al, 1996). Two HPLC systems are used for the project to examine the CSF IgG oligosaccharides as follows.

2.2.5.1. System 1: GlycoSep-N™ column

A normal phase system using a GlycoSep-N™ column comprises a polymeric silica matrix with bonded amide functional groups. Oligosaccharides are adsorbed by hydrogen bonding and are sequentially eluted by increasing the polarity of the mobile phase. Separation is due mainly to the hydrodynamic volume (size) of the oligosaccharide, which is due to monosaccharide composition, but is also affected by arm specificity and linkage of the units (Guile et al, 1996).

In conjunction with the IgG glycans, a series of dextran hydrolysates is run to calibrate the peak retention times in terms of glucose units (gu values). Gu values can then be assigned to the peaks for the oligosaccharides and used to identify their structures. The Oxford Glycobiology Institute have compiled an extensive table of gu values, both measured and calculated, for *N*-glycans for this particular HPLC system (see Appendix).

2.2.5.2. System 2: Wax column

This is a reverse phase system using a weak anion-exchange stationary phase. Oligosaccharides are separated according to overall charge, which is due to the number of attached sialic acids. Degree of sialylation is determined by comparison with sialylated oligosaccharides released from fetuin and run in conjunction as calibrators.

2.2.6. Deglycosylation

Deglycosylation of glycoproteins is used in the project in two instances: a) removal of carbohydrate from anti-IgG used as capture antibody in the glycoprotein lectin immunoassays, to obviate non-specific binding by the lectins; b) removal of carbohydrate from a CSF sample with oligoclonal IgG and comparing bands after isoelectric focusing with bands in the untreated sample, to ascertain if oligoclonal banding can be attributed to carbohydrate differences. For each of these it is desirable to remove as much carbohydrate as possible, with minimal destruction of peptide. A number of deglycosylation methods are available, as follows.

2.2.6.1. Chemical methods.

The use of sodium periodate to oxidize sugar chains has been described in section 2.1.1.1. Conditions can be chosen so that damage to the peptide is minimized: use of low temperature (0 to 4°C) so that secondary reactions are reduced; protection from light to prevent ozone production, and using minimum concentration of periodate and incubation time (Marshall and Neubauer, 1972). Concentration of periodate used varies between 0.005 and 0.1 M. The method would appear to be suitable for deglycosylation of capture antibody since intact saccharides are not required. A method is described using 0.1 M $NaIO_4$ at 4°C for 3 days for an antibody-lectin immunoassay of α-fetoprotein (Kinoshita et al, 1989).

Hydrazinolysis has been described in section 2.1.1.2 as a means of releasing *N*-glycans for analysis. However, peptide is denatured and not suitable for use in analysis.

Trifluoromethanesulphonic acid (TFMS) can be used as a deglycosylation agent for both *N*- and *O*-linked oligosaccharides (Sojar and Bahl, 1987). However, recovery of peptide may be as little as 70% in some glycoproteins (quoted by Oxford GlycoSystems for their product) and TFMS has been found not to remove the terminal GlcNAc of

some glycoproteins (Tams and Welinder, 1994) or sialylated chains of others (Raju and Davidson, 1994).

2.2.6.2. Enzymic methods

The use of enzymes to release oligosaccharides from glycoproteins is described in section 2.1.2.1. The most suitable enzyme to use for removal of *N*-glycans from glycoproteins is peptide-*N*-glycosidase F, which cleaves *N*-glycans at their link to asparagine. Thus, as long as steric hindrance does not prevent the enzyme from accessing the oligosaccharides, complete removal of intact glycans should be possible. The enzyme has no adverse effect on the peptide, so the method is highly suitable for deglycosylation of capture antibody (Köttgen et al, 1993) as well as studying protein glycosylation, and is used in the project to deglycosylate oligoclonal IgG prior to isoelectric focusing.

For complete deglycosylation of most glycoproteins, prior denaturation is recommended in order to allow the enzyme access to the oligosaccharides (Mann et al, 1994; Hirani et al, 1987; see also Oxford GlycoSystems application sheet for peptide-*N*-glycosidase F). This normally involves heating the glycoprotein at 100°C in the presence of up to 1%($^{w}/_{v}$) sodium dodecyl sulphate. However there is little information specifically on the treatment of human IgG.

2.3. Samples for Project

Cerebrospinal fluid used in the project was leftover specimen after routine analysis of samples from patients who had undergone a lumbar puncture at Hurstwood Park Neurological Center. Permission to use this waste material was obtained from the Princess Royal Hospital Research Ethics Committee. Permission to review patients' notes was also obtained from the committee, and from the consultant neurologists concerned.

Generally the CSF samples were stored for up to one week at 4°C before research analysis. However treatment, storage and selection of samples varied at different stages of the project, and will be described in the relevant methods sections.

2.4. Statistical Methods and Computer Programs

The statistics in this thesis, paired and unpaired Student's t-tests and Pearson correlation analysis, were produced using Prism (version 3.00) from GraphPad Software Inc., San Diego, CA, USA. Graphs were produced also using Prism as well as Excel 97 from Microsoft Corp., USA. Word-processing was performed using Word 97 from Microsoft Corp.

Chapter Three

Development of an IgG Immunoassay

The primary aim of producing an assay for IgG was to provide a basis for the development of the glycoprotein lectin immunosorbent assays to probe the oligosaccharides of CSF IgG. However, creation of a reliable, highly sensitive assay for IgG would also be useful for quantitation of the low levels of IgG that are found in CSF samples.

The first step was to conjugate anti-IgG antibody with the isoluminol derivative, ABEI, which was then used, along with assay tubes coated with anti-IgG as capture antibody, to set up a sandwich-type chemiluminescent immunoassay. This was then optimised and evaluated, ready for the modification with biotinylated lectins.

3.1. ABEI-Conjugation of Anti-IgG

3.1.1. Introduction

After the conjugation reaction between anti-IgG and ABEI, the reaction mixture was subjected to ion-exchange chromatography to separate viable conjugate from unbound components. The eluate was collected as fractions, whose viability was tested by incubation in IgG-coated tubes to enable selection of fractions for pooling.

3.1.2. Reagents and Equipment

1. Water used throughout was deionized by reverse osmosis and ion exchange chromatography.
2. Affinity purified anti-human IgG, γ-chain specific, 1 mg/mL, developed in

goat (product no. I-3382); human IgG (supplied as 5.7 mg/mL) (I-2511); "high avidity" BSA (300 g/L solution) (A3424) and phosphate buffered saline (PBS) tablets: obtained from Sigma Chemical Co., Poole, Dorset, BH12 4QH. PBS was prepared by dissolving one tablet in 200 mL deionized water to produce a solution of 0.01 M phosphate buffer, pH 7.4.

3. LIAMAT 300 luminescence immunoassay analyser (the 'LIA') made by Byk-Sangtec Diagnostica GmbH & Co., KG Von-Hevesy-Strasse, 63128, Dietzenbach, Germany. Supplied by Cambridge Life Sciences plc, Ely, Cambs. CB7 4DT.
4. 'Star' assay tubes, polystyrene, 5 mL, 12 x 75 mm, (code 115075). Greiner Labortechnik Ltd., Brunel Way, Stroudwater Business Park, Stonehouse, Glos., GL10 3SX.
5. Aminobutylethylisoluminol-ethylene glycol-*bis*-(succinic acid)-*N*-hydroxysuccinamide ester (ABEI-EGS), HPLC purified, was supplied courtesy of Cambridge Life Sciences plc, stored at -20°C in acetonitrile in 10 µL aliquots, with an effective ABEI concentration of 3.4 nmol/µL.
6. Sephacryl S-300 Superfine ion-exchange resin (40-105 µm wet bead diameter), obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden, packed in a 30 cm x 2 cm column.
7. Fraction Collector 270 (Chemlab U.K.), used with a Varioperpex II pump (LKB/Pharmacia, Uppsala, Sweden).
8. Luminescence 'Starter' reagents. Reagent 1: one Perhydrit tablet (500 mg peroxide) (Merck Cat No 7201, 0100) in 50 mL M NaOH. Reagent 2: 1.0 mg microperoxidase (sodium salt from equine heart cytochrome c) (Sigma M6756) in 50 mL deionized water.
9. Teleostean fish gelatine 45% solid. Product no. G7765, Sigma Co.

10. Marvel™. Milk powder, food item
11. Sucrose. Granulated sugar, food item.
12. Blocking solution: Marvel (0.2 g) + granulated sugar (0.5 g) in 10 mL coating buffer.
13. Coating buffer (0.1 M): prepared by dissolving 4.2 g $NaHCO_3$ in 500 mL deionized water (solution A), 1.06 g Na_2CO_3 in 100 mL deionized water (solution B) and adding B to 500 mL of A to produce pH 9.2.
14. Conjugate buffer: PBS containing 100 g/l BSA (Sigma A3424) and 10 g/L NaN_3.
15. Wash buffer: phosphate buffered saline with gelatine (PBS-G). To 1.0 litre PBS was added 200 mg sodium azide and 0.2 mL fish gelatine
16. All other chemicals not specified above were of Analar grade and obtained from Merck Ltd. Poole, Dorset.

3.1.3. Methods

3.1.3.1. Addition of ABEI-EGS to Anti-IgG

The effective molar reaction ratio of ABEI-EGS to anti-IgG is 30:1. The solvent:water ratio of the mixture should be between 1:4 and 1:10 (recommendations from Cambridge Life Sciences).

Concentration of anti-IgG = 1 mg/mL. This is 6.67 nmol/mL, assuming molecular weight of anti-IgG to be 150 kDa. Supplied ABEI-EGS is 34 nmol per 10 µL aliquot, therefore anti-IgG required for reaction is 1.13 nmol, i.e. 169 µL. This would give a solvent:water ratio of 10:169, i.e. 1:17. Therefore add 30 µL acetonitrile to give a ratio of 40:169, i.e. 1:4.2.

169 µL of anti-IgG was placed in a plastic cuvette. To this was added 30 µL acetonitrile and mixed, then 10 µL of ABEI-EGS and mixed. This was incubated for 2 h at RT.

3.1.3.2. Column Separation

The Sephacryl S-300 column was flushed with 0.01 M PBS and the flow-rate set to 0.6 mL/min. The fraction cutter was set to 100 s per fraction, giving a volume of 1.0 mL per fraction. The reaction mixture was introduced to the top of the column and, using 0.01 M PBS for elution, 50 fractions of 1.0 mL were collected.

3.1.3.3. Assay of Fractions.

a. Total counts

10 µL from every fraction from numbers 6 to 50 was diluted with 990 µL of PBS. Then 10 µL aliquots of these dilutions were pipetted to the bottom of LIA assay tubes in duplicate and the RLUs measured.

b. Bound Counts

42 µL of human IgG (1.0 mg/mL) was mixed with 22 mL coating buffer and 0.2 mL of this was placed into each of 100 assay tubes and incubated overnight at RT. After washing each tube three times with 1.5 mL PBS-G and aspirating, tubes were incubated with 0.3 mL blocking solution for 2 h, then washed.

Each fraction was diluted with PBS-G to produce a count (see 3.1.3.4. RLU measurement) of approximately 100 000 per 10 µL.

In triplicate, 10 µL of each diluted fraction was placed in an IgG-coated tube with 200 µL of PBS-G. Tubes were incubated for 1.5 h at RT, then washed and aspirated as above, and the RLU measured as below. Pooled fractions of viable conjugate were mixed 9:1 with conjugate buffer and stored at -70°C in 20 µL aliquots.

3.1.3.4. RLU Measurement

The LIA was used in luminometer mode only. After final washing, tubes loaded in racks were passed into the luminometer, where, for each tube, 300 µL of each starter reagent was automatically injected, forcibly to mix thoroughly, and the luminescence reading was taken in a 0.2 s window (RLU reading).

3.1.4. Results for ABEI-Conjugation of Anti-IgG

Measurement of the column fractions is shown in Fig 3.1. The plot of the total RLU of each fraction (left-hand y axis) shows ABEI to be eluted as two peaks: ABEI-anti-IgG conjugate, and free ABEI. The % Bound plot (right-hand axis) shows binding of the viable conjugate to the IgG of the coated tube, expressed as a percentage of the activity (100 000 RLU) of the diluted fraction added to the tube. The viable conjugate is seen to be eluted as the first peak. Based on these results, fractions 10, 11 and 12 were pooled and stored in 20 µL aliquots at -70°C.

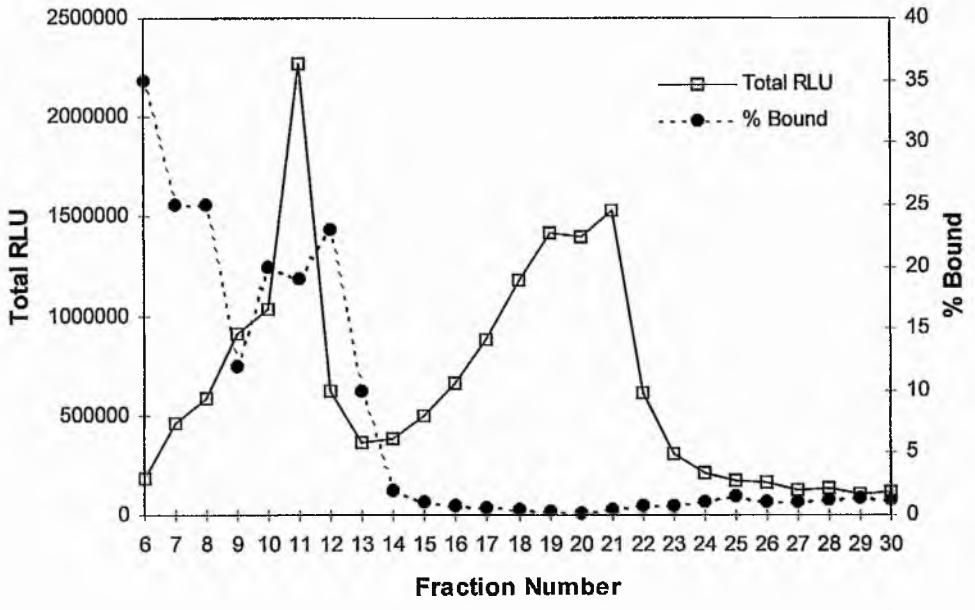


Figure 3.1. Measurement of fractions from the Sephacryl column after the conjugation reaction between ABEI-EGS and anti-IgG.

3.1.5. Discussion

This is a straightforward procedure for conjugating anti-IgG with isoluminol. However, this was the second attempt at ABEI-anti-IgG conjugation. With the first attempt the peak for the bound fraction was not well defined and it was difficult to select fractions to pool, producing a conjugate that gave low binding in the assays. This time, however, the bound and free peaks were well defined. Fractions 6 to 9, although showing high %binding, have total RLUs that are not part of the total RLU peak and these fractions were not included in the pool. The frozen conjugate remained effective for at least 1 year, producing over 40% maximum binding in the assays.

3.2. Optimization of IgG Immunoassay

3.2.1. Introduction

Using the prepared ABEI-anti-IgG conjugate, a basic immunoassay was set up which was then optimised by varying the conditions and reagent preparation.

Unless stated otherwise, all washing steps consisted of three additions of 1.5 mL of wash solution with aspiration after each, and all incubations were at room temperature.

3.2.2. Reagents

1. Reagents and equipment were as described previously, with the following additions.
2. Dynagel: hydrolysed pig gelatine: kindly donated by Dr Hans Hager, Cambridge Life Sciences.
3. Dextran: Average m.w. 10 kDa. Prod. No. 9260, Sigma Co.
4. Tween 20. Prod. No. 66368, Merck Ltd.
5. Saline-Tween wash solution. 9 g NaCl in 1 L deionized water containing 0.5 mL Tween 20.

3.2.3. Methods

3.2.3.1. Preparation of a Calibration Curve

Anti-IgG was diluted 1:200 with coating buffer (30 µL in 6 mL buffer). 200 µL was pipetted into each of 30 assay tubes and incubated for 8 h at RT then washed x3 with 1.5 mL PBS-G and aspirated. Tubes were blocked for 2 h with 1 g/L BSA in PBS, 300 µL per tube, and washed and aspirated as before. As controls, three anti-IgG coated tubes were not blocked.

Calibration solutions were prepared by diluting the supplied IgG to produce a solution of 100 mg/L (0.1 mL with 5.6 mL PBS-G). This was then further diluted as shown in Table 3.1. Then 200 µL of PBS-G was pipetted into the assay tubes, followed by 10 µL of calibration solution to labelled tubes in triplicate and incubated for 1 h at RT. The 1.0 mg/L calibrator was added to the non-blocked control tubes. Tubes were washed and aspirated as above, then conjugate diluted 1:900 (4 µL + 3.6 mL PBS-G) was added to the tubes (including 'total' tubes which were not subsequently aspirated) and incubated for 1 h at RT. Blank tubes were prepared with PBS-G instead of conjugate. After further washing as above, the RLU of each tube was determined as described in section 3.1.3.4.

Table 3.1. Preparation of calibration solutions.

Volume 'stock' 100 mg/L IgG (µL)	Volume PBS-G (µL)	[IgG] (mg/L)
40	160	20
20	180	10
10	190	5
10	400	2
80	20	0.8
60	40	0.6
40	60	0.4
20	80	0.2
10	90	0.1
10	190	0.05
10	490	0.02
0	200	0

3.2.3.2. Variation of Coating Antibody Dilution

Anti-IgG was diluted 1:200 with coating buffer (100 µL in 20 mL buffer). Then taking 3 mL of this and double diluting with 3 mL additions of coating buffer, solutions of 1:400, 1:800 and 1:1600 were prepared. Tubes were coated with each of these solutions, incubating 200 µL per tube for 12 h, then washing and aspirating x3.

Standards of 0, 0.05, 1.0 and 20.0 mg/L IgG were prepared from a stock 100 mg/L IgG solution as described in section 3.2.3.1. 10 µL of these with 200 µL of PBS-G were pipetted into sets of coated tubes in triplicate and incubated for 1 h at RT. After washing and aspirating, 200 µL of conjugate diluted 1:900 was added to each tube and incubated for a further 1 h at RT. Tubes were again washed and aspirated and RLUs measured. Blank and 'total' tubes were prepared as described in 3.2.3.1.

3.2.3.3. Trial of Different Blocking Agents

a. Uncoated tubes

Fish gelatine, Dynagel, Dextran and BSA were diluted in PBS to produce 10 g/L and 1 g/L solutions. 1.0 mL volumes of these solutions were placed in plain polystyrene assay tubes in triplicate and incubated for 4 h at RT. After washing x3 with 1.5 mL PBS-G and aspirating, 0.2 mL of conjugate diluted 1:500 with PBS was pipetted into each tube and incubated for 16 h at RT. Tubes were washed and aspirated as above and the RLUs obtained as described in section 3.1.3.4.

b. Coated Tubes

Anti-IgG was diluted 1:200 with coating buffer and 0.2 mL pipetted into each of 30 assay tubes to coat for 8 h at RT, then washed and aspirated. A range of blocking agents in PBS was prepared: 1g/L BSA (0.3 mL of 300 g/L BSA in 100 ml PBS); 5 g/L BSA; 10 g/L BSA; 1 g/L BSA with 1 mL/L Tween; 1 g/L BSA with 5 mL/L Tween; 1 g/L BSA with 10 mL/L Tween; PBS containing 20 g/L Marvel with 50 g/L sucrose. For each blocker,

0.3 mL was added to coated tubes in triplicate. No blocker was added to 3 coated tubes. Tubes were incubated for 1 h at RT then washed and aspirated.

ABEI-anti-IgG conjugate was diluted 1:1000 with PBS and 200 µL was added to each tube, also to 3 uncoated tubes and 3 tubes which were not further treated ('totals'). Tubes were incubated for 16 h at RT then washed and aspirated and RLU measured.

3.2.3.4. Variation of Sample Incubation Time

Tubes were coated with 200 µL of anti-IgG diluted 1:200 with coating buffer, incubated for 2 h, washed, aspirated and blocked with 200 µL of PBS containing 1 g/L BSA and 1 mL/L Tween. IgG standards solutions of 0, 0.5, 1.0, 3.0 and 5.0 mg/L were prepared from a stock 100 mg/L IgG solution as described in 3.2.3.1 and 200 µL PBS-G and 10 µL of standard solutions were pipetted into duplicate assay tubes. Tubes were incubated for 0.5, 1, 2, 4 or 6 h, then washed and aspirated. Conjugate was diluted 1:1500 and 200 µL was added to each tube, incubated for 1 h at RT, washed and aspirated and the RLU measured. Blank and 'total' tubes were prepared as before.

3.2.3.5. Variation of Conjugate Incubation Time

Tubes were coated for 12 h with anti-IgG diluted 1:200 with coating buffer, washed and aspirated then blocked for 2 h with 200 µL of 1 g/L BSA and 1 mL/L Tween in PBS, washed and aspirated. IgG standards of 0, 0.5, 1, 3 and 5 mg/L were prepared as described in section 3.2.3.1. Then 200 µL of PBS-G was added to each coated tube followed by 10 µL standard solution in duplicate in six sets. Tubes were mixed, incubated for 2 h at RT, then washed and aspirated.

Conjugate was diluted 1:1500 and 200 µL added to each tube and to 'total' tubes which were not further diluted. PBS-G was added to 'blank' tubes. Sets of tubes were incubated for 0.5, 1, 2, 4 or 6 h.

One-step assays were also tried. 200 µL of diluted conjugate was added to two sets of

coated tubes immediately followed by 10 µL of IgG standards. One set was incubated for 2 h and the other for 4 h.

After the designated incubation times, all sets of tubes were washed x3 with PBS-G (one set, incubated for 6 h, was washed with saline-Tween) and, along with 'total' and blank tubes, RLUs were measured.

3.2.3.6. Variation of Anti-IgG Coating Incubation Time

Anti-IgG was diluted 1:200 with coating buffer and used, 200 µL per tube, to coat tubes for 2 h, 4 h and 16 h. Tubes were washed with PBS-G, except one set incubated for 16 h was washed with saline-Tween.

IgG standard solutions of 0, 0.5, 1.0, 3.0 and 5.0 mg/L were prepared as described in 3.2.3.1. Into coated tubes was pipetted 200 µL of PBS-G followed by 10 µL of standards in duplicate. Tubes were incubated for 1 h, then washed with PBS-G, or one set with saline-Tween. Conjugate was diluted 1:1800 in PBS-G and 200 µL added to each tube and incubated for 2 h. Tubes were washed in PBS-G (saline-Tween for one set) and RLU measured.

3.2.3.7. Comparison of Saline-Tween and PBS-G Washes

30 tubes were coated with 0.2 mL of anti-IgG diluted 1:200 with PBS for 16 h then washed with PBS-G and aspirated. IgG standard solutions of 0, 0.2, 0.6, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/L were prepared as described in 3.2.3.1. To coated tubes was added 200 µL PBS-G then 10 µL of standards in two sets in duplicate. Tubes were incubated for 1.5 h, then one standard set was washed x3 with PBS-G and one standard set washed x3 with saline-Tween. 200 µL of conjugate diluted 1:1800 was added to tubes (plus 'totals') and incubated for 2 h, then tubes were again washed with PBS-G or saline-Tween, and RLU measured.

3.2.3.8. Storage of IgG Standards

IgG standard solutions from 0 to 5 mg/L were prepared from stock 100 mg/L IgG standard as described in section 3.2.3.1. From these, 0.5 mL aliquots were stored a) at -20°C for 1 month, b) at 4°C for 2 weeks in polystyrene analyser cups, c) at 4°C for 2 weeks in glass bottles. Before use the aliquots were thawed and/or mixed and, together with a set of standards freshly prepared from stock 100 mg/L IgG, were assayed as described in 3.2.3.7, using saline-Tween washing.

3.2.4. Results

3.2.4.1. Results for Preparation of a Calibration Curve

Figure 3.2 shows a plot of the IgG calibrators after subtraction of the baseline (mean of RLUs, i.e. 3733, for 0 mg/L IgG calibrator). Mean count for 'totals' was 111179; mean for blanks was 507.

The assay appears to be viable, with a projected working range for IgG in the region of 0.1 to 5.0 mg/L.

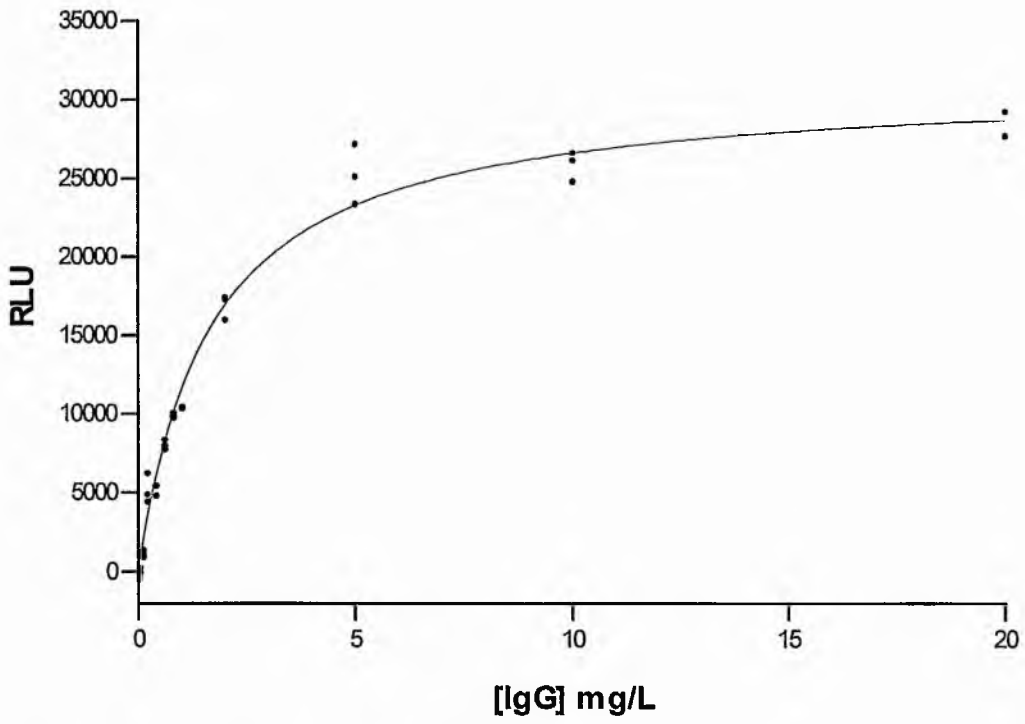


Figure 3.2. Preliminary plot of IgG standards after baseline subtraction.

3.2.4.2. Results for Variation of Coating Antibody Dilution

Figure 3.3 shows RLUs plotted for the different coating antibody dilutions after subtraction of the baselines (i.e. mean RLUs for the 0 mg/L IgG solutions). At the 20 mg/L IgG level there is a decrease in binding with increase in coating antibody dilution. However, at the 1.0 mg/L level, the binding for the 1:200 dilution is reduced and highest binding is shown by the 1:400 dilution. As the working range of the assay will probably be in the region of 0.1 to 5.0 mg/L, a 1:400 dilution of coating antibody might be most suitable.

3.2.4.3. Results for Trial of Different Blocking Agents

a. Uncoated tubes

Binding of the anti-IgG conjugate to uncoated tubes is shown in Figure 3.4. BSA, at either dilution, is shown to be the most effective blocker.

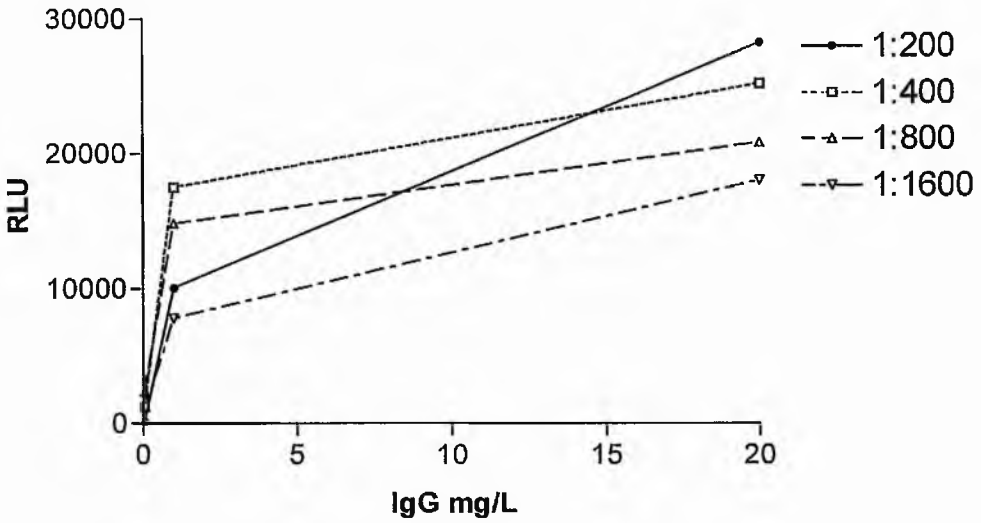


Figure 3.3. IgG binding with different dilutions of coating antibody.

b. Coated Tubes

Non-specific binding of the conjugate with different blocking agents is shown in Figure 3.5. Marvel/sucrose is shown to be the most effective blocker, followed by 1 g/L BSA with 1 mL/L Tween. BSA without Tween, however, increases rather than blocks non-specific binding and is therefore unsuitable as a blocking agent.

3.2.4.4. Results for Variation of Sample Incubation Time

Figure 3.6 shows IgG binding with different incubation times for the sample. Binding is virtually complete by 2 h.

3.2.4.5. Results for Variation of Conjugate Incubation Time

Figure 3.7 shows that binding of conjugate is almost complete by 4 h. With 5 mg/L IgG, at 4 h incubation of conjugate, the mean RLU was 44381 compared to 115146 for the 'totals' i.e. 38% of the added conjugate was bound to captured sample IgG. The binding of conjugate at 2 h incubation was still high, with an RLU of 34564 at the 5 mg/L level, i.e. 30% of conjugate bound.

With the 4 h assay saline-Tween washing gave very similar results to PBS-G washing, suggesting that it might be possible to replace PBS-G with saline-Tween washing in the IgG assay, which would be more convenient and cheaper. The one-step assays show flattened curves compared to the two-step assays, with higher binding up to 2 mg/L IgG sample, but reduced binding above this. It is possible that when increased levels of sample IgG is added it forms a complex with the conjugate but this is not binding to the capture anti-IgG of the tube coating. It is possible that a one-step assay could be developed, but it might have a reduced linear range.

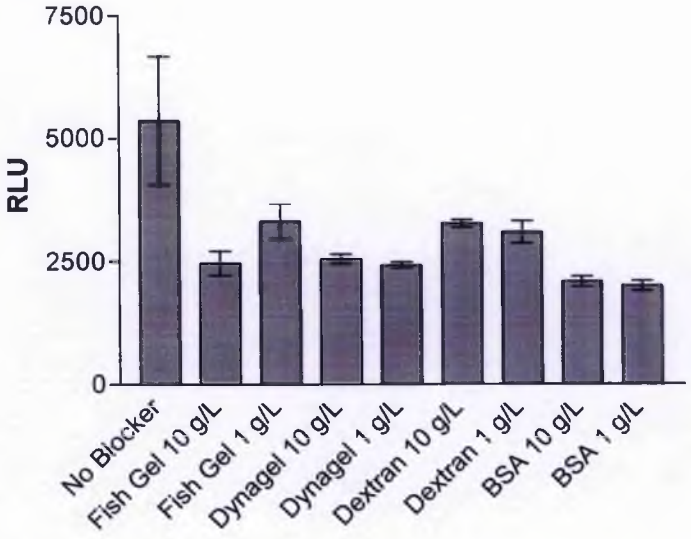


Figure 3.4. Anti-IgG-ABEI conjugate binding to uncoated tubes. All blockers made up in 0.01 M PBS. Error bars show standard error of the mean of triplicate tubes.

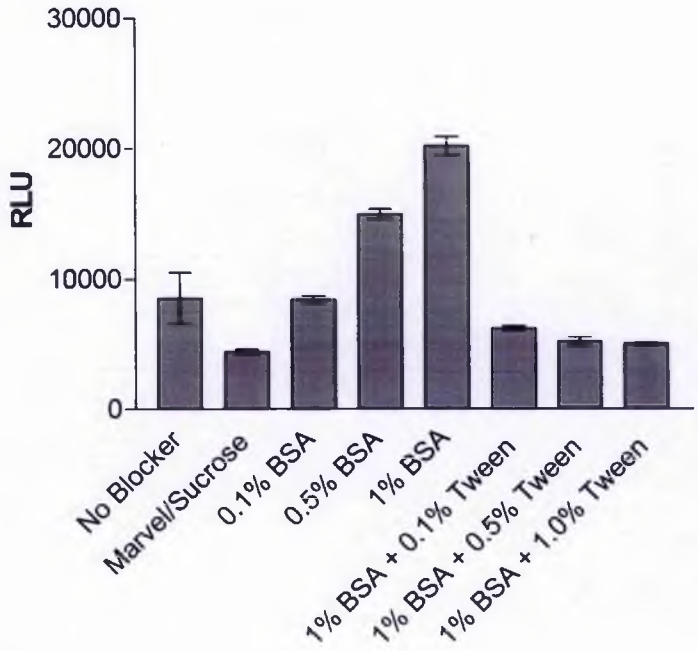


Figure 3.5. Non-specific binding by anti-IgG-ABEI conjugate to antibody-coated tubes with different blocking agents. Error bars show standard error of the mean of triplicate tubes. All blockers made up in 0.01 M PBS. Marvel/Sucrose: 20 g/L Marvel + 50 g/L sucrose.

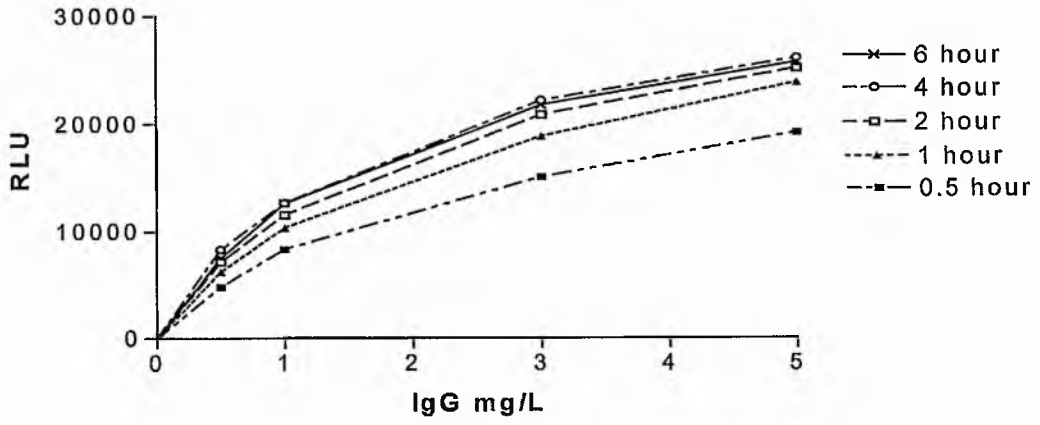


Figure 3.6. Binding of IgG standard solutions with different sample incubation times.

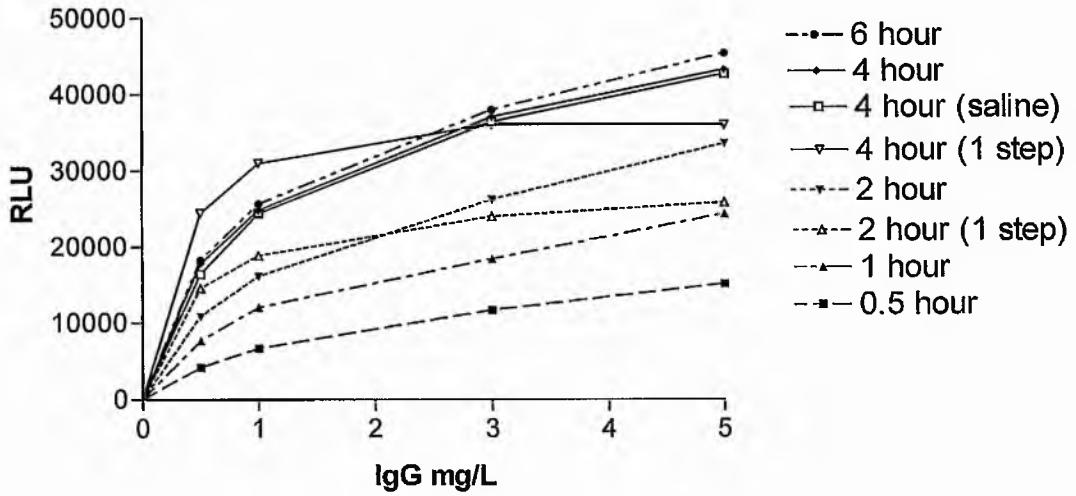


Figure 3.7. Measurement of IgG with different incubation times of anti-IgG-ABEI conjugate, and with trials of one-step assays and of using a saline-Tween wash.

3.2.4.6. Results for Variation of Anti-IgG Coating Incubation Time

Figure 3.8 shows that anti-IgG coating is achieved with an incubation time as short as 2 h. For the times tested, percentage binding with the 5 mg/L IgG standard ranged from 20% to 22%. Results for the 4 h incubation are lowest: for reasons unknown the non-specific binding (i.e. binding for 0 mg/L IgG) was higher than for the other times. Washing with saline-Tween does not appear to have an adverse effect.

3.2.4.7. Results for Comparison of Saline-Tween and PBS-G Washes

It can be seen from Figure 3.9 that washing with saline-Tween or PBS-G makes very little difference to either the binding potential or the precision of the assay.

3.2.4.8. Results for Storage of IgG Standards

Fig 3.10 shows that highest IgG binding is obtained if standards are freshly prepared from stock 100 mg/L IgG solution. Standards prediluted and stored at 4°C for 2 weeks show a loss of IgG in the low concentrations, presumably through adhesion of IgG to the sides of the container. Storage in polystyrene shows even greater loss, whether stored at 4°C or -20°C.

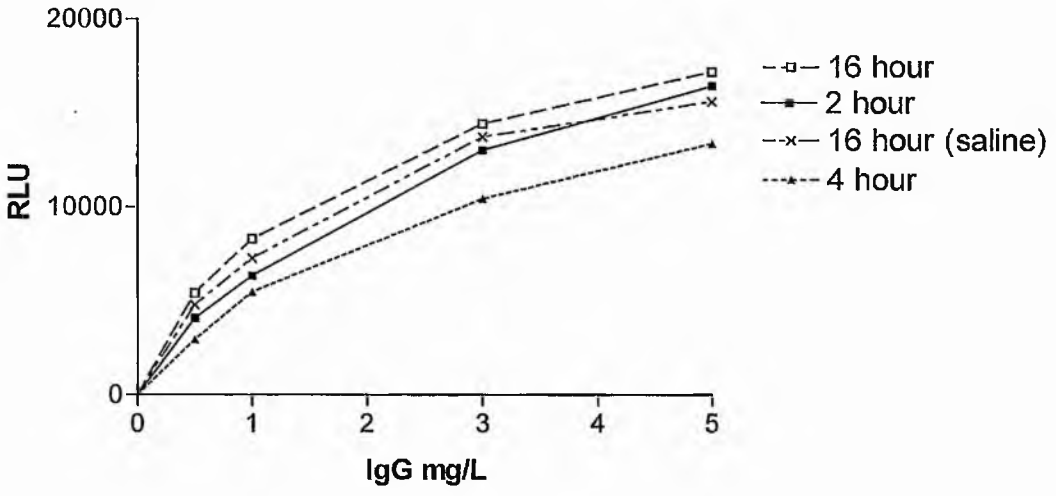


Figure 3.8. Variation of anti-IgG coating time. Apart from one 16 h incubation, washing was with PBS-G.

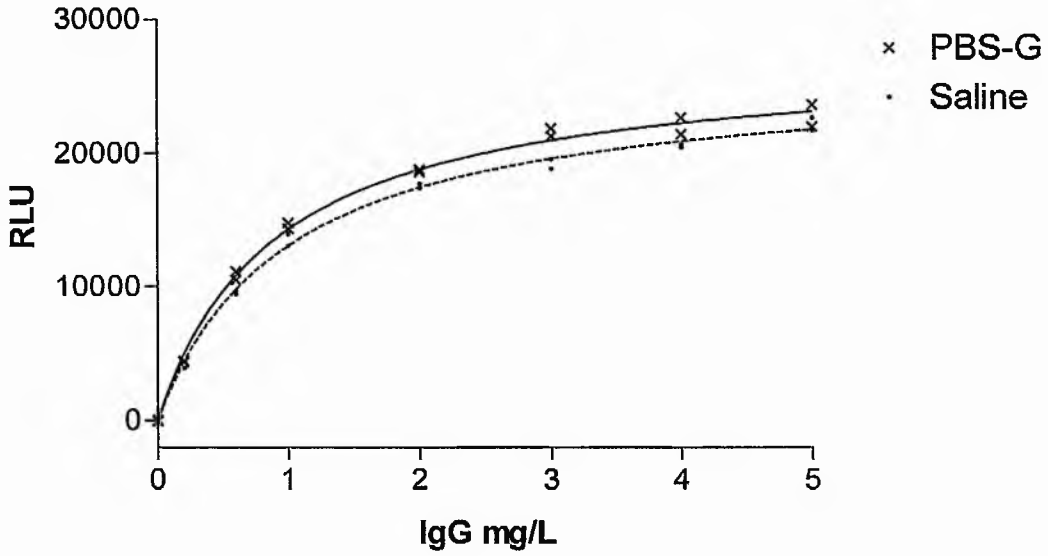


Figure 3.9. Comparison of washing with saline-Tween (9 g/L NaCl containing 0.5 ml/L Tween 20) and PBS-G (0.01 M phosphate buffered saline, pH 7.4, containing 0.1 g/L fish gelatine).

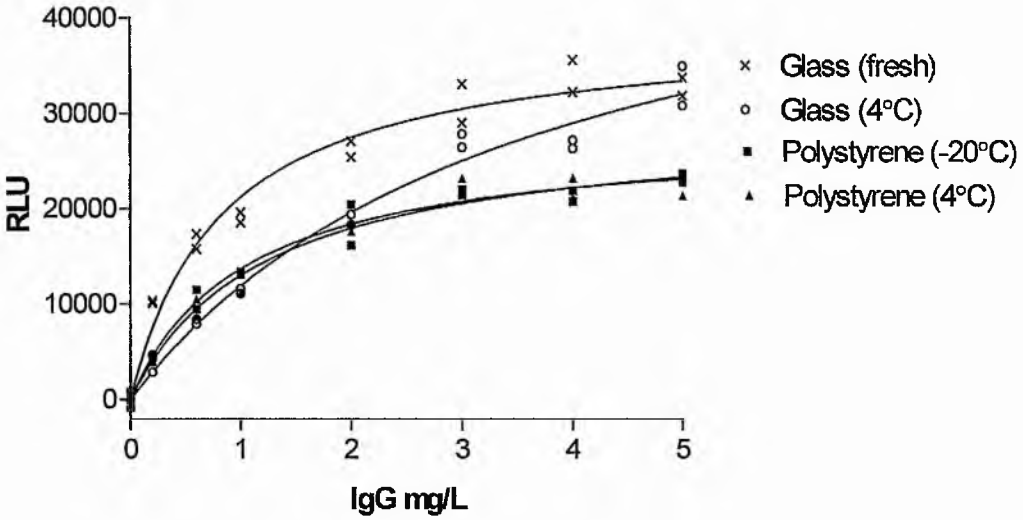


Figure 3.10. Storage of IgG standards.

3.2.5. Discussion

The results show that an IgG assay can be produced with a working range from less than 1 mg/L to about 5 mg/L IgG. Sensitivity is particularly high for low levels of IgG and it should be possible to have the range as low as 0.02 mg/L. At the upper end of the range, with the flatter calibration curve greater imprecision was found, and in later assays wider differences were found between replicates for standards and patients' samples. Therefore an upper limit of 4 mg/L IgG was chosen for the assay. With levels of IgG ranging from 20 to 40 mg/L in normal CSF, and up to 1000 mg/L in disease states, samples will need to be diluted 40 fold or more, if the sample volume remains at 10 μL.

IgG standard solutions will be prepared freshly before use from stock 100 mg/L IgG solution. Storage, whether at 4°C or -20°C leads to loss of IgG, probably to the sides of the containers, but should be minimised if IgG is stored as a stock solution with high IgG concentration.

The preparation of anti-IgG coated tubes is straightforward and sufficient coating can be achieved with an incubation time as short as 2 h. The antibody can be used at a 1:400 dilution, but diluting more than this gives less binding of IgG. Experiments with different blocking agents show that Marvel/sucrose is most effective, however it was decided not to use this as the sugars would probably interfere in the lectin assays. BSA (1 g/L) with Tween (1 mL/L), found to be the next most effective blocker, will be used. BSA is not highly glycosylated and should not interfere in the lectin assays.

Sample incubation time can be reduced to 1 or 2 h without significant loss of IgG binding. Incubation of anti-IgG-ABEI conjugate should be for a minimum of 2 h.

Saline-Tween can be used as washing solution throughout tube coating and assay procedures in place of PBS-G, thus reducing expense and preparation time, important in view of the large volumes of wash solution that are used.

Initial trial of a one-step assay showed potential, but this will not be pursued further, as a

two-step procedure is needed for development of the lectin-binding assays.

3.3. Evaluation of the Assay

3.3.1. Introduction

The working range for the assay was decided at 0.02 mg/L to 4 mg/L IgG. CSF samples would be diluted 1:40 to bring the IgG into this range, with further dilution for the occasional sample that exceeded this range. A number of assays were performed to evaluate the method.

3.3.2. Reagents

- Unless mentioned below, reagents were as previously described.
- Coating buffer, 0.1 M. Prepared by dissolving 4.2 g $NaHCO_3$ in 500 mL deionized water (solution A), 1.06 g Na_2CO_3 in 100 mL water (solution B) and adding B to 500 mL of A until pH was 9.2.
- Assay buffer: Tween 20 (0.5 mL/L) in 0.01 M PBS.
- Wash solution ("Saline-Tween"): 9 g/L NaCl containing 0.5 mL/L Tween 20.
- Blocker: 1 g/L BSA with 1 mL/L Tween 20 in 0.01 M PBS.
- Immunoturbidimetric assay of CSF IgG was performed using the method of Cowdrey (1990) using the Cobas-Bio centrifugal analyser (Roche Diagnostica, Welwyn Garden City, Herts, AL7 3AY.

3.3.3. Methods

Washing at all stages consisted of 3 additions of 1.5 mL saline-Tween with aspiration after each addition.

3.3.3.1. The Working IgG Method

Tubes were coated overnight (16 h) with 200 µL anti-IgG (diluted 1:400 with coating buffer). After washing, they were incubated for 2 h with 300 µL blocker, then rewashed.

Assay buffer (200 µL) was added, followed by 10 µL of freshly prepared IgG calibrators in duplicate, or 10 µL in duplicate of CSF samples diluted 1:40 with PBS immediately beforehand, and the tubes were incubated for 1.5 h at RT. The tubes were further washed before adding 200 µL ABEI-anti-IgG conjugate (2 µL in 30 mL assay buffer), and incubating at RT, in the dark, for 2 h. After final washing, the tubes were loaded in racks and fed into the luminometer, where automatically, 300 µL of each starter reagent was injected into each tube, and the luminescence reading taken in a 2 s window (RLU reading).

Patients' CSF samples were stored for up to 4 weeks at 4°C, and had already been assayed routinely by the laboratory, including IgG measurement by IT.

3.3.3.2. Imprecision and Recovery Studies.

Three CSF samples, with low, medium and high levels of IgG, were measured 15 times each in a batch. Thirty random CSF samples were measured after 0, 5 or 15 µL additions of 1 g/L IgG to 200 µL aliquots of CSF, to produce spiking of 0, 24.4 and 69.8 mg/L IgG.

3.3.3.3. Correlation Study

Seventy CSF samples, chosen at random, were assayed by the described chemiluminescent immunoassay, and results compared with those obtained by routine IT IgG measurement.

3.3.4. Results

3.3.4.1. Results for the Working IgG Method

A typical non-linear calibration curve is shown in Figure 3.11. The limit of detection, calculated as 2 SDs from zero of results for 0 mg/L IgG (n = 10), was 0.02 mg/L. This is equivalent to 0.8 mg/L IgG in CSF after multiplication by the dilution factor (x40).

3.3.4.2. Results for Imprecision and Recovery Studies

Table 3.2 shows data for within batch imprecision and recovery studies. The CVs were between 4.3% and 6.6% for the imprecision, and the recoveries were close to 100%.

3.3.4.3. Results for Correlation Study

Correlation of the method with immunoturbidimetry (IT) was good (r = 0.88) as shown by measurement of 68 samples (Figure 3.12). Another two samples, with IgG values of 440 mg/L and 489 mg/L obtained by IT (after dilution), were diluted 1:200 before measuring by LIA, producing results of 500 and 620 mg/L respectively. These results have not been plotted, but if included in the correlation, would increase r to 0.99.

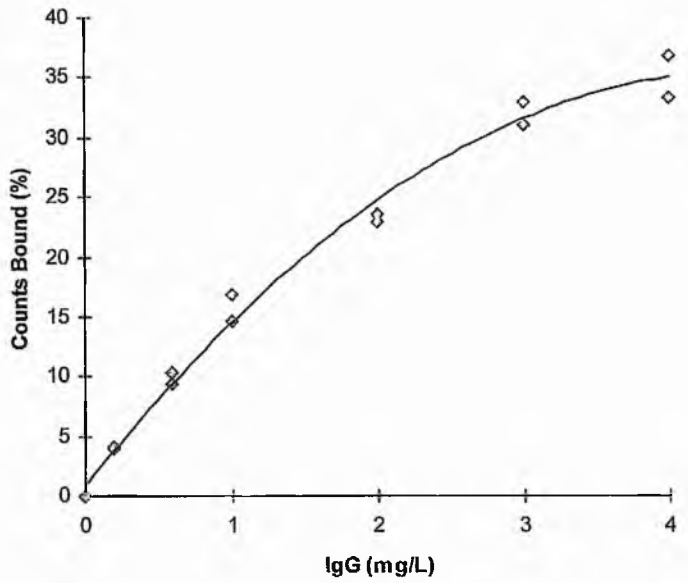


Figure 3.11. A typical calibration curve for the IgG chemiluminescence immunoassay. $y = -1.7x^2 + 15.38x + 0.85$; r = 0.99.

Table 3.2. Imprecision and recovery studies for CSF IgG immunoassay.

Sample	Mean ± SD	n	CV%
Imprecision			
Low IgG CSF	6.3 mg/L ± 0.27	15	4.3
Medium IgG CSF	18.5 mg/L ± 1.22	15	6.6
High IgG CSF	64.5 mg/L ± 3.80	15	5.9
%Recovery			
Addition of 24.4 mg/L IgG	106% ± 10.7	30	10.1
Addition of 69.8 mg/L IgG	97% ± 11.7	30	12.1

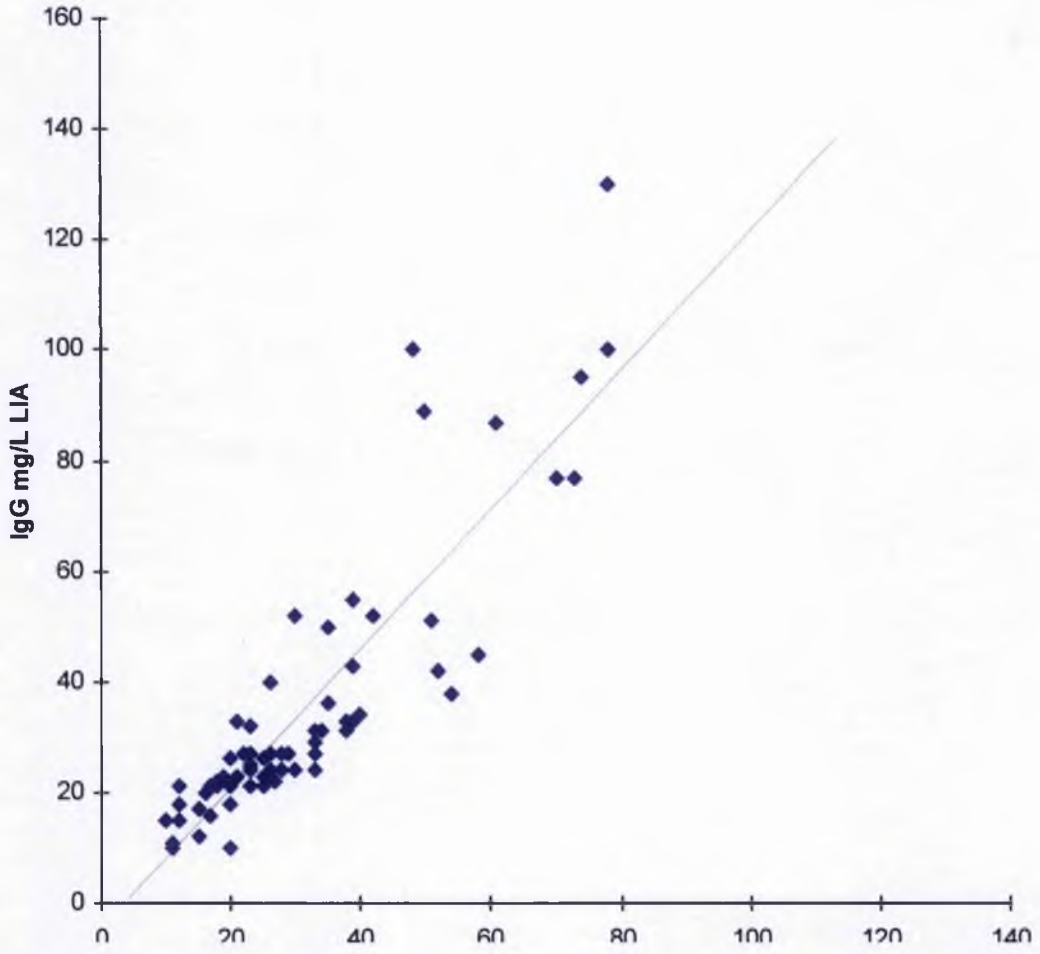


Figure 3.12. IgG measured in 68 CSF samples by both chemiluminescence immunoassay and immunoturbidimetry. $y = 1.26x - 4.35$; $r = 0.88$.

3.3.5. Discussion

A useful method for measurement of IgG in CSF has been developed, with a working range of 0 to 4 mg/L IgG. Pre-dilution of CSF is required. The feature of the assay is its sensitivity, with limit of detection as low as 0.8 mg/L, after correction for dilution, and potentially as low as 0.02 mg/L if samples are not pre-diluted.

The assay is robust and shown to be precise, with true measurement of IgG in CSF. The method correlated well with an IT method using not only different calibration material, but also a different antibody. The IgG of the calibrators was derived from human serum, CSF IgG not being commercially available, but results suggest commutability of the method.

3.4. Development of an IgG Immunoassay: Overall Discussion

Using the working method described, it takes about 4 hours to measure a batch of 40 CSF samples. This is more rapid than RID or electrophoretic techniques, but with sample pre-dilution and two pipetting stages, it is not as rapid as an automated one-step IT assay. Attempts were made to automate the assay using the LIA analyser, but problems were encountered with carryover, probably stemming from the LIA's main use of routine analysis of serum samples. Despite thorough washing through of the analyser before research use, contamination persisted and this line of development was eventually abandoned. Frequent mechanical failures of the LIA were also a hindrance, as well as difficulties in fitting in research work around routine use of the analyser.

The calibration range of this sensitive method, with 1:40 pre-dilution of CSF samples is 8 to 160 mg/L - wider than with IT (10 to 100 mg/L: Cowdrey, 1991). The detection limit, of 0.8 mg/L IgG with pre-dilution of sample, is lower than other methods. The detection limit of RID is 30 mg/L, which is the upper margin of the normal range for CSF. Rate

nephelometry, like IT, measures down to 10 mg/L (Christenson et al, 1998).

The anti-IgG-ABEI conjugate was easy to prepare and stable for 1 year, and probably longer, at -20°C. Storage in small aliquots, e.g. 20 μL, ensured that fresh, active conjugate was available for every assay.

Although a method for measurement of IgG in CSF is presented here, there is potential for measuring IgG in other body fluids. For measuring IgG in serum, where a less sensitive assay is required, it should be feasible to construct a one-step competitive-binding immunoassay. The same anti-IgG-coated tubes would be used, but the anti-IgG-ABEI conjugate would be added at the same time as diluted serum (or undiluted CSF). This would produce a more rapid method. There is the capability of full automation in either format.

With the high sensitivity of this method, there is the capacity, using the same coated tubes, to measure IgG subclasses, by substitution of anti-IgG-ABEI conjugate with anti-IgG subclass-ABEI conjugate. It might also be possible to develop assays for specific IgG antibodies e.g. serological tests for viral antibodies. The next chapter will describe incorporation of lectins into the assay, to enable detection of carbohydrate variants of IgG.

Chapter Four

Development of Lectin Binding Assays for CSF IgG

4.1. Introduction

Most of the development of the lectin binding assays was carried out using Con A and then extended to the other lectins. The conversion of the IgG immunoassay to incorporate lectins was not as straightforward as anticipated and, as will be seen, a number of problems were encountered and various ways of overcoming them were tried. Finally, a range of lectin binding assays was used to probe the oligosaccharides of IgG in CSF samples from a group of MS patients and a group of control patients.

For the formats of the immunoassays described in this chapter please refer to Figure 2.5 in Chapter 2 (section 2.2.2.6). The lectin immunoassays all follow scheme 3 [Figure 2.5(c)]. The IgG immunoassays follow scheme 1 [Figure 2.5(a)], apart from the intermediate IgG assay described in the first experiment (*4.2.3.1. Use of Biotin-Streptavidin in the IgG immunoassay*) which follows scheme 2 [Figure 2.5(b)].

4.2. Incorporation of Con A in Immunoassay

4.2.1. Introduction

This section will show how the IgG immunoassay was modified with a biotin-streptavidin system, and then extended to using biotinylated Con A. Different incubation times for Con A were tried and various blocking agents were tested.

4.2.2. Reagents

Reagents are as described previously, with the following additions.

1. Anti-human IgG, biotinylated; γ-chain specific; raised in goat, affinity purified, and lyophilised. Product no. BA-3080, Vector Laboratories Ltd., 16 Wulfric Square, Bretton, Peterborough, PE3 8RF, U.K.
2. Isoluminol-streptavidin: 3.5 moles of isoluminol per 1 mole of streptavidin. Contains 0.1 mg protein (by Biuret). Product no. S8532, from Sigma Chemical Co., Poole, Dorset, BH12 4QH. Made up with 1 mL PBSG and stored at -70°C in 50 µL aliquots.
3. Concanavalin A, biotinylated, 5 mg. B-1005, Vector Laboratories. Reconstituted in 1.0 mL water, to give 10 mM HEPES buffer, 0.15 M NaCl. Stored at -70°C in 50 µL aliquots.
4. D-xylose. Analar reagent, product no. 10372, Merck Ltd, Hunter Boulevard, Magna Park, Lutterworth, Leics., LE17 4XN.

4.2.3. Methods

Washing at all stages consisted of 3 additions of 1.5 mL of saline-Tween, with aspiration after each addition. All incubations were at room temperature.

4.2.3.1. Use of Biotin-Streptavidin in the IgG Immunoassay

The usual IgG immunoassay [scheme 1, Figure 2.5(a)] was compared to a modified IgG immunoassay using biotinylated anti-IgG and streptavidin-isoluminol [scheme 2, Figure 2.5(b)] as follows.

Anti-IgG capture antibody was diluted 1:200 in coating buffer, and 200 µL was added to assay tubes and incubated for 9 hours, then washed. IgG solutions were prepared as described in section 3.2.3.1. 200 µL of PBSG was pipetted into the antibody-

coated tubes, followed by 10 µL of IgG standards in duplicate tubes. Two series of standards were prepared. All tubes were incubated for 1 h then washed.

Following scheme 1, anti-IgG-ABEI conjugate was diluted 1:1000 with PBSG and 200 µL was added to one series of tubes, incubated for 1 h, washed and the RLU measured for each tube.

Following scheme 2, biotinylated anti-IgG was diluted 1:1000 with PBSG and 200 µL was added to the other series of tubes, incubated for 1 h then washed. Streptavidin-isoluminol was diluted 1:2000 with PBSG, 200 µL was added to each tube, incubated for 1 h, washed, then the RLU measured.

4.2.3.2. Blockers of Non-Specific Binding with Con A

The following solutions were prepared in carbonate buffer: 10 g/L and 1 g/L BSA, 10 g/L fish gelatine, 10 g/L dextran, 10 g/L pig gelatine, 10 g/L D-xylose, 10 g/L and 1 g/L human serum albumin (HSA), 10 ml/L Tween and 10 g/L BSA containing 10 ml/L Tween. Also 10 g/L BSA was prepared in PBS.

400 µL of each solution (made freshly in glass bottles) was pipetted into assay tubes in duplicate. Duplicate tubes were also prepared with PBS only, carbonate buffer only and with no solution. All tubes were incubated for 1 h, then washed.

Biotinylated Con A was diluted 1:5000 with PBS, 200 µL was added to each tube and incubated for 1 h then washed. Streptavidin-isoluminol was diluted 1:10 000 with PBS, 200 µL was added to each tube, incubated for 30 min, washed and RLUs measured.

4.2.3.3. Con A Incubation Times

Replicate assays (scheme 3) of IgG standard solutions were performed, with a different incubation time of Con A for each.

Tubes were coated overnight with 300 µL of anti-IgG diluted 1:400, washed, then blocked overnight with 400 µL of 10 g/L BSA in PBS and washed.

IgG standard solutions of 0 mg/L, 1 mg/L, 5 mg/L and 100 mg/L were prepared according to the protocol in section 3.2.3.1. A CSF sample was diluted 1:40 and 1:200 with PBS. 200 μL of PBS containing 0.2 g/L BSA was pipetted in the assay tubes. This was followed by 10 μL of 0 mg/L or 5 mg/L IgG solutions in duplicate tubes, or 10 μL of CSF or CSF dilutions in duplicate tubes. Also included were, in duplicate, plain tubes, anti-IgG coated tubes without blocker and blocked tubes without anti-IgG coat, which had no sample added.

All tubes were incubated for 2 h and washed. 200 μL of biotinylated Con A diluted 1:5000 in PBS containing 0.2 g/L BSA was pipetted into tubes and incubated for 5 min, 15 min, 30 min, 1 h, 2 h, 3 h or 4 h. The CSF samples and unblocked or uncoated tubes were incubated for 30 min.

After washing, 200 μL of isoluminol-streptavidin diluted 1:20000 in PBS containing 0.2 g/L BSA was pipetted into all tubes, incubated for 20 min, washed and RLUs measured.

4.2.4. Results

4.2.4.1. Results of Use of Biotin-Streptavidin in the IgG Immunoassay

Figure 4.1 compares the IgG assay using biotin-streptavidin with the assay using anti-IgG-ABEI. Use of biotin-streptavidin appears viable and adaptation with biotinylated lectin looks feasible.

4.2.4.2. Results for Blockers of Non-Specific Binding with Con A

Figure 4.2 shows that the most effective blocking agent with Con A was BSA with little difference between the 10 g/L and 1 g/L solutions. However use of Tween with the BSA reduced blocking ability.

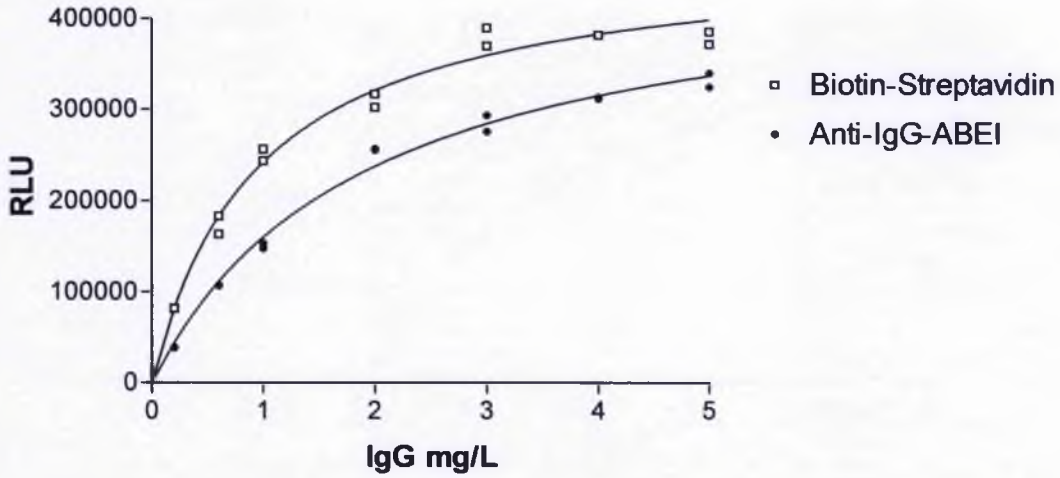


Figure 4.1. Comparison of an IgG assay using biotinylated anti-IgG and isoluminol-streptavidin with one using anti-IgG-ABEI. Duplicate points are shown for the standards for each assay. See 4.2.3.1 for details of method.

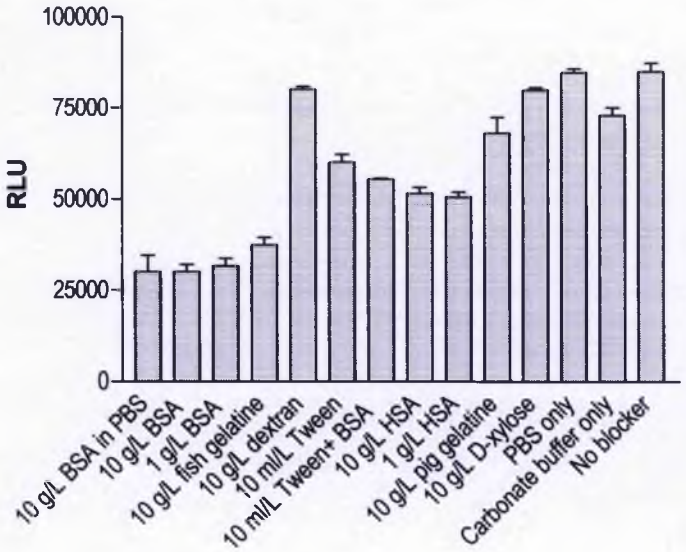


Figure 4.2. Solutions blocking Con A binding to assay tubes. Prepared in carbonate buffer, pH 9.4, unless specified as PBS. Error bars represent SEM of duplicate samples. See 4.2.3.2 for details of method.

4.2.4.3. Results for Con A Incubation Times

Figure 4.3 shows that binding of Con A at the 5 mg/L IgG level increases with incubation time up to a maximum at 3 or 4 h. However, binding of Con A without addition of sample IgG (i.e. 0 mg/L IgG) is as high as binding with 5 mg/L IgG at all incubation times. It appears that Con A is binding to the capture anti-IgG coated to the tube as well as to bound sample IgG. There is also high binding to the 'no anti-IgG' tube (i.e. no antibody coating, but blocked), which is undesirable.

Figure 4.4 compares the binding at several levels of sample IgG with 30 min incubation of Con A. Although the binding increases from 0 to 1 and to 5 mg/L, non-specific binding at 0 mg/L is relatively high and presents a problem. Also the RLU reading for the 1:200 dilution of CSF is similar to the 1:40 dilution and the neat samples, again indicating non-specific binding.

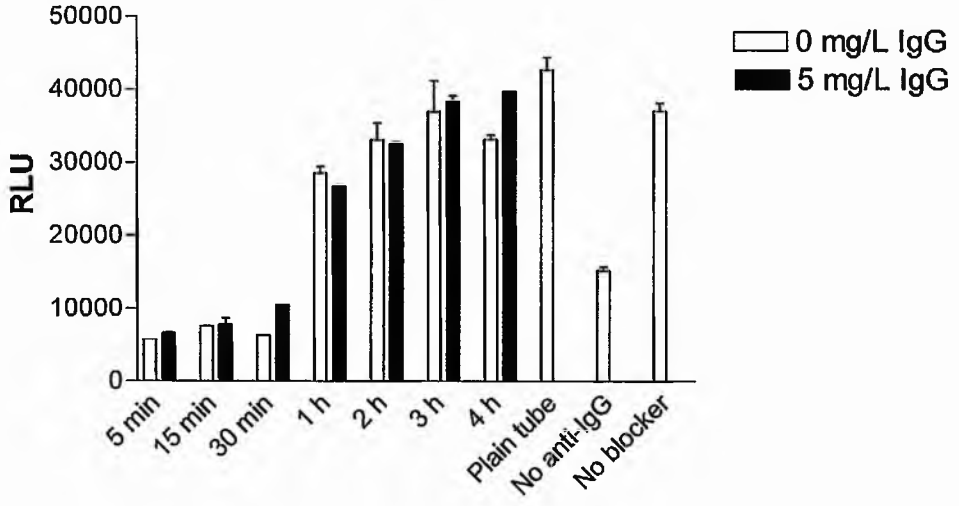


Figure 4.3. RLUs measured with 0 and 5 mg/L IgG samples with a range of Con A incubation times. Error bars represent SEM of duplicate samples. See 4.2.3.3 for details of method.

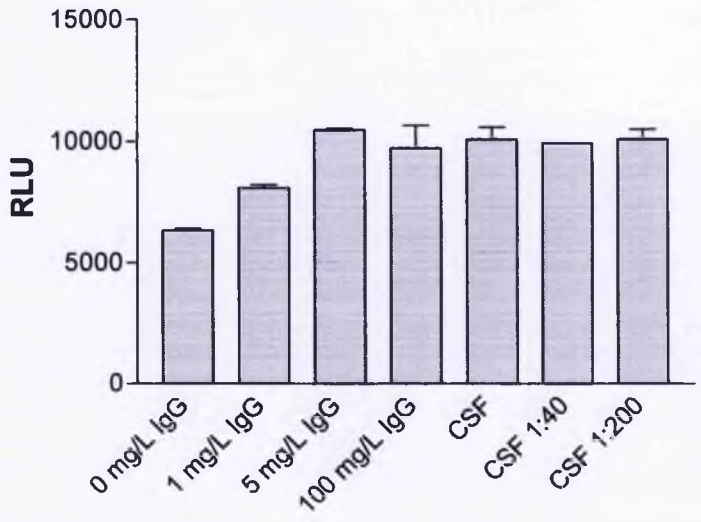


Figure 4.4. Measured RLU with different IgG solutions, CSF and diluted CSF with 30 min incubation of Con A. Error bars represent SEM of duplicate samples. See 4.2.3.3 for details of method.

4.2.5. Discussion for Incorporation of Con A

A relatively large amount of non-specific binding by Con A was found, which is probably due to binding to carbohydrate on the anti-IgG capture antibody. Therefore, either an aglycosylated form of antibody could be used, if available, or the existing antibody could be deglycosylated.

4.3. Deglycosylation of Capture Antibody

4.3.1. Introduction

It was decided to deglycosylate the capture antibody in use, and to do this after binding the antibody to the assay tube, using a chemical method - i.e. oxidation with sodium periodate - rather than an enzymic method. It will be shown how, after an initial trial assay using periodate, reaction conditions were varied to obtain as complete deglycosylation as possible with least damage to antibody activity. The method was compared to two recently published protocols for the deglycosylation of capture antibody using stronger concentrations of periodate (Baharaki et al, 1996; Keusch et al, 1996).

Despite deglycosylation of capture antibody, high blank readings persisted. It was suspected that periodate oxidation might be producing active groups such as aldehydes which were binding to a sample constituent or to a reagent, and ethanolamine was introduced to neutralise such groups. The Con A inhibitor, α-methylpyrannoside was used to ascertain if the lectin was binding specifically or non-specifically to carbohydrates.

Finally a range of lectins was used to assess the glycosylation of IgG in four CSF samples.

4.3.2. Reagents

As described previously, with the following additions

1. Citric acid ($C_6H_8O_7.H_2O$). Product no. 10081, Merck Ltd.
2. Disodium hydrogen phosphate (Na_2HPO_4). Product no. 10248, Merck Ltd.
3. Citrate/phosphate buffer, 50 mM, pH 4.0.

   Reagent A: dissolve 1.05 g citric acid in 100 mL water.

   Reagent B: dissolve 1.79 g Na_2HPO_4 in 100 mL water.

   Add A (approximately 31 mL) to B (approximately 24 mL) to give pH 4.0.
4. Sodium m-periodate ($NaIO_4$). Product no. S-1147, Sigma Co. To prepare 0.1M $NaIO_4$, dissolve 0.214 g in 10 mL citrate/phosphate buffer, pH 4.0, immediately before use.
5. Ethanolamine. $CH_2(OH)CH_2NH_2$. Merck, 10325. Mw 61.08. Supplied as 10% (^{v}/v) solution in water. A 0.1 M solution was prepared by diluting 1.5 mL in 25 mL PBS.
6. Tris buffered saline (TBS). Dissolve one tablet (T-5030, Sigma Co.) in 15 mL water to produce a 0.05 M Tris buffer, pH 7.6, containing 0.15 M sodium chloride.

7. Coating buffer. Now prepared from capsules from Sigma (product no C-3041). Contents of one capsule are dissolved in 100 mL water to produce a 0.05 M carbonate-bicarbonate buffer, pH 9.6.
8. Lectins, biotinylated. From Vector Laboratories. Kit I (product no. BK-1000) containing 1 mg quantities of biotinylated Con A, DBA, PNA, RCA_{120}, SBA, UEA 1 and WGA. Kit III (BK-3000) containing 0.5 mg quantities of biotinylated DSL, ECL, GSL II, Jacalin, LEL, STL and VVL. Also biotinylated SNA, 2 mg (B-1305). All lectins reconstituted with 0.5 mL water. **CAUTION: RCA_{120} is mildly toxic.**
9. Lectin assay buffer, containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. Dissolve 10.15 g of $MgCl_2.6H_2O$ (29096, Merck), 7.35 g of $CaCl_2.2H_2O$ (10070, Merck) and 25 mg NaN_3 in 50 mL water. Add 45 µL of this solution, plus 23 µL Tween 20, to 45 mL TBS.
10. PBS-T. PBS containing 0.5 mL/L Tween 20.
11. α-Methylpyrannoside (methyl-α-D-glucopyrannoside). $C_7H_{14}O_6$. Mw 194.2. M-9376, Sigma Co.

4.3.3. Methods

All washing steps are with 3 additions of 1.5 mL of Saline-Tween, with aspiration after each.

4.3.3.1. Initial Trial of Periodate-Treated Tubes.

20 tubes were coated overnight with 200 µL of anti-IgG, diluted 1:400 in coating buffer, then washed. The tubes were blocked with 300 µL of BSA (10 g/L) for 1 h, then washed. To all tubes, apart from 4, was added 400 µL 0f 0.1 M sodium periodate, followed by incubation overnight at 4°C in the dark (i.e. in a refrigerator), then washed.

Solutions containing 100 mg/L, 5 mg/L, 1 mg/L and 0 mg/L of IgG were prepared as described in section 3.2.3.1. 200 µL of PBSG was pipetted into the coated tubes followed by 10 µL of IgG solution (0, 1, 5 and 100 mg/L IgG solutions in periodate-treated tubes, in duplicate; 0 and 100 mg/L solutions in untreated tubes, in duplicate) and incubated for 90 min. After washing, 200 µL biotinylated Con A, diluted 1:1000 with lectin assay buffer, was added to each tube, with further 90 min incubation. After washing tubes were incubated for 60 min with 200 µL isoluminol-streptavidin diluted 1:1000 in PBS-T, then washed and RLUs measured.

4.3.3.2. Effect of Temperature and Concentration of Periodate

80 tubes were coated overnight with 200 µL of anti-IgG diluted 1:400, then blocked with 300 µL of 10 g/L BSA for 1 h. Solutions of periodate of 0.1 M (0.214 g sodium periodate/10 mL citrate/phosphate buffer pH 4.0) and 0.01 M (0.021 g/10 mL citrate/phosphate buffer pH 4.0) were made freshly and kept at 4°C. 400 µL amounts of either periodate solution were added to tubes, in duplicate, at different times, incubated at 25°C or 4°C, for between 1 h and 18 h, then all tubes were washed together.

A CSF sample containing 80 mg/L IgG (CSF 'B') was diluted with PBS (25 µL in 20 mL), and 200 µL of this was added to all tubes and incubated for 90 min. After washing, to half the tubes 200 µL of biotinylated Con A (diluted 1:1000) was added and incubated for 90 min, washed and 200 µL of isoluminol-streptavidin was then added, incubated for 60 min, washed and RLUs measured. To the other tubes, 200 µL of anti-IgG-ABEI (diluted 1:15000) was added, incubated for 2 h, washed and RLUs measured.

4.3.3.3. Increasing Incubation Time with 0.01 M Periodate.

50 tubes were coated and blocked as in section 4.3.3.2. 0.01 M periodate was prepared freshly (42 mg/ 20 mL citrate buffer) and 0.4 mL was added to all tubes at the same time which were incubated at 4°C and washed at varying times.

A CSF sample containing 41 mg/L IgG (CSF 'C') was diluted with PBS (40 µL in 8 mL) and 0.2 mL was added to coated tubes which were incubated for 90 min and washed. Tubes were assayed for [IgG] or for Con A binding as in section 4.3.3.2.

4.3.3.4. Comparison of Periodate Treatments

100 tubes were antibody coated and blocked as in 4.3.3.2. Also 60 tubes were treated with blocker only (300 µL of 10 g/L BSA for 1 h).

Immediately before use, 0.1 M periodate solution was prepared by diluting 428 mg of sodium periodate in 20 mL citrate/phosphate buffer. 5 mL of this was diluted with 5 mL citrate/phosphate buffer to produce 0.05 M periodate solution. 4 mL of 0.1 M periodate was diluted with 36 mL citrate/phosphate buffer to produce 0.01 M periodate.

20 coated tubes were incubated with 0.3 mL 0.1 M periodate for 3 min.

20 coated tubes were incubated with 0.3 mL 0.05 M periodate for 10 min.

20 coated tubes and 20 'blocked only' tubes were treated with 0.01 M for 20 h at 4°C.

20 coated tubes were not periodate treated.

A CSF sample (CSF 'VR') with an IgG concentration of 27 mg/L was diluted to give a solution of 5 mg/L IgG (200 µL CSF + 880 µL PBS). Then dilutions were made from this to give solutions containing 1 mg/L and 3 mg/L of IgG.

a. IgG Assay

200 µL of PBS-T and then 10 µL of 0 mg/L, 1 mg/L, 3 mg/L or 5 mg/L CSF IgG solutions was pipetted in duplicate into labelled periodate untreated tubes. This was

repeated with 3 min periodate treated tubes, 10 min treated tubes and 20 h treated tubes. All tubes were incubated for 2 h, washed and 300 µL of anti-IgG-ABEI (diluted 1:10 000) was added. After 2 h incubation, tubes were washed and RLUs measured.

b. Lectin Binding

200 µL of PBS-T was pipetted into 9 periodate untreated tubes and into 9 tubes each of the three periodate treatments. Into 6 tubes of each treatment set was also pipetted 10 µL of 5 mg/L CSF IgG. All tubes were incubated for 1.5 h then washed.

0.2 mL of Con A, diluted 4 µL in 10 ml lectin assay buffer, was pipetted into triplicate tubes of each treatment set without the CSF addition and into triplicate tubes with the CSF IgG addition. 0.2 mL of RCA, diluted 10 µL in 10 mL of lectin assay buffer, was pipetted into triplicate tubes of each sample set with the CSF IgG addition. Tubes were incubated for 1.5 h and washed.

0.2 mL of isoluminol-streptavidin, diluted 2 µL in 20 mL PBS-T, was pipetted into all tubes, incubated for 1 h, washed and RLUs measured.

4.3.3.5. Dilution of Capture Antibody

Although a 1:400 dilution of the capture antibody was found to be optimum for tube coating for the IgG assay, the dilution for the lectin assay, where the coating is subject to periodate treatment, was now tested.

A 1:100 dilution of anti-IgG was made by adding 60 µL to 6 mL of coating buffer, then by doubling dilutions, using 3 mL amounts of coating buffer, 1:200, 1:400 and 1:800 solutions were prepared. Tubes were coated overnight with 0.2 mL of each solution, then blocked with 0.3 mL of 10 g/L BSA for 1 h, and treated with 0.01 M sodium periodate for 20 h at 4°C.

IgG standard solutions were prepared and IgG assays performed as described in 3.3.3.1. using the different sets of coated tubes.

4.3.3.6. Use of Ethanolamine and a Con A Inhibitor

20 tubes were coated with 200 µL anti-IgG at 1:400 for 21 h and washed. 300 µL of 0.01 M periodate (0.21 g in 10 mL citrate buffer) was added to the tubes and incubated for 21 h and washed. 400 µL of 0.1 M ethanolamine solution was added to 14 coated tubes; 0.4 mL of PBS was added to other tubes and incubated for 21 h then washed. 300 µL BSA (0.45 mL of 300 g/L BSA in 15 mL coating buffer) was added to coated tubes and to 20 untreated tubes and incubated for 1 h then washed.

200 µL of PBS was added to all tubes, then 10 µL CSF sample in duplicate (no CSF was added to one series of tubes), and incubated for 1.5 h and washed. Con A was diluted with lectin assay buffer (2 µL in 20 mL) without BSA. To 10 mL of this was added 0.4 g α-methylpyrannoside (to give a 200 mM solution). 200 µL of diluted Con A or 200 µL of diluted Con A with inhibitor was added to tubes and incubated for 1 h then washed. 200 µL isoluminol-streptavidin diluted 1 µL in 10 mL lectin assay buffer (no BSA) was added to each tube and incubated for 30 min then washed and RLUs were measured.

4.3.3.7. Analysis of IgG Glycosylation Using a Panel of Lectins

200 tubes were coated overnight with 200 µL of anti-IgG diluted 1:400 (105 µL in 42 mL coating buffer) then washed. To each tube and to 170 additional plain tubes was added 0.3 mL of blocker (3.6 mL of 300 g/L BSA in 120 mL PBS). All tubes were incubated for 2 h and washed. 400 µL of 0.01 M periodate was then added to all tubes and incubated for 20 h at 4°C then washed.

a. Total IgG Assay

IgG calibration solutions of 5, 3, 2, 1, 0.6, 0.2 and 0 mg/L were prepared as described in section 3.2.3.1. Four CSF samples, A, B, C, and D were all diluted 1:40 (10 µL CSF + 390 µL PBS). They were also diluted to give an IgG concentration of 10

mg/L, according to Table 4.1. To labelled coated tubes was added 200 µL of PBS-T then 10 µL of diluted CSF or calibrator and incubated for 2 h and washed. 200 µL of anti-IgG-ABEI was added to the tubes, incubated for 2 h, washed and RLUs measured.

b. Lectin Immunoassay

For the lectin assays, the CSF dilutions containing 10 mg/L IgG (Table 4.1) were sampled. At this level of IgG, the capture antibody would be virtually saturated by IgG, ensuring that the same amount of IgG for all samples is available for lectin binding. 200 µL of each diluted CSF (or 200 µL of PBS for 'no CSF' tubes) was added to 32 coated tubes each and 32 blocked only tubes each and incubated for 2 h then washed. Different biotinylated lectins were diluted 1:1000 (5 µL in 5 mL lectin assay buffer) and 200 µL of this (or 200 µL of lectin assay buffer only for 'no lectin' tubes) was added in duplicate to tubes for CSFs A to D and 'no CSF' tubes. After incubating for 2 h and washing, 200 µL of diluted isoluminol-streptavidin (8 µL in 80 mL PBS-T) was added to all tubes, incubated for 40 min, washed and RLUs measured.

4.3.4. Results

4.3.4.1. Results for Initial Trial of Periodate-Treated Tubes

Figure 4.5 shows that there is an improvement in background binding with periodate treated tubes, although it is still higher than would be liked. Differentiation between sample IgG concentrations also needs to be increased.

Table 4.1. Details of CSF samples used in total IgG and lectin assays.

CSF	Oligoclonal Bands	IgG mg/L by IT	Dilution to produce 10 mg/L IgG. mL CSF	mL PBS
A	Positive	22	0.33	0.40
B	Positive	72	0.10	0.62
C	Negative	25	0.33	0.50
D	Negative	17	0.50	0.35

4.3.4.2. Results for Effect of Temperature and Concentration of Periodate

Figures 4.6(A) and 4.6(B) show the effect of 0.1 M and 0.01 M periodate respectively on the activity of the capture antibody (i.e. 'IgG' in the legend) and on the glycosylation of the antibody (i.e. 'Con A' in the legend). Figure 4.6(A) shows that 0.1 M periodate effectively destroys most carbohydrate on the antibody after about 3 h, however deterioration in the activity of the antibody also begins after a few hours even at 4°C. Destruction of the anti-IgG activity at room temperature is considerable. On the other hand, 0.01 M periodate [Figure 4.6(B)] shows a gentler reaction and there is only a small reduction in anti-IgG binding up to 18 h incubation, by which time most of the carbohydrate appears to be eliminated.

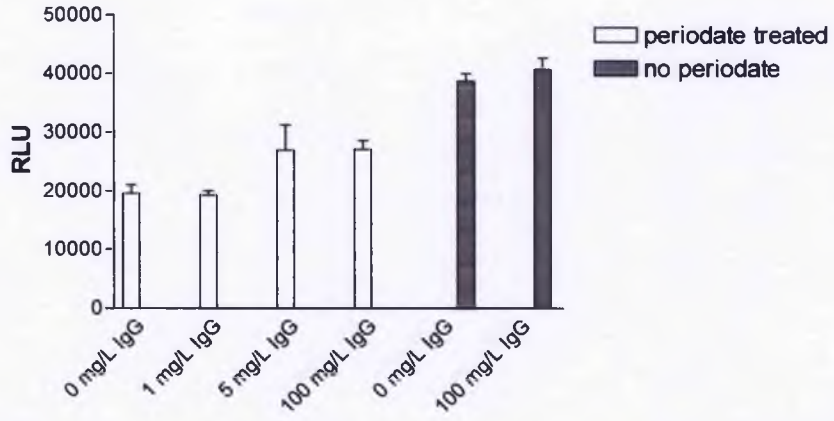


Figure 4.5. Initial trial of 0.1 **M** periodate treated tubes. Error bars represent SEM for duplicate samples. See 4.3.3.1 for details of method.

4.3.4.3. Results for Increasing Incubation Time with 0.01 M Periodate

Figure 4.7 shows that the integrity of the capture antibody is maintained with up to 27 h incubation with 0.01 M periodate at 4°C. Deglycosylation, shown by Con A binding, is almost complete by about 9 h. Binding of Con A to carbohydrate in the sample IgG can be clearly seen (i.e. the legend 'Con A + CSF') after the pretreatment with periodate has deglycosylated the capture antibody.

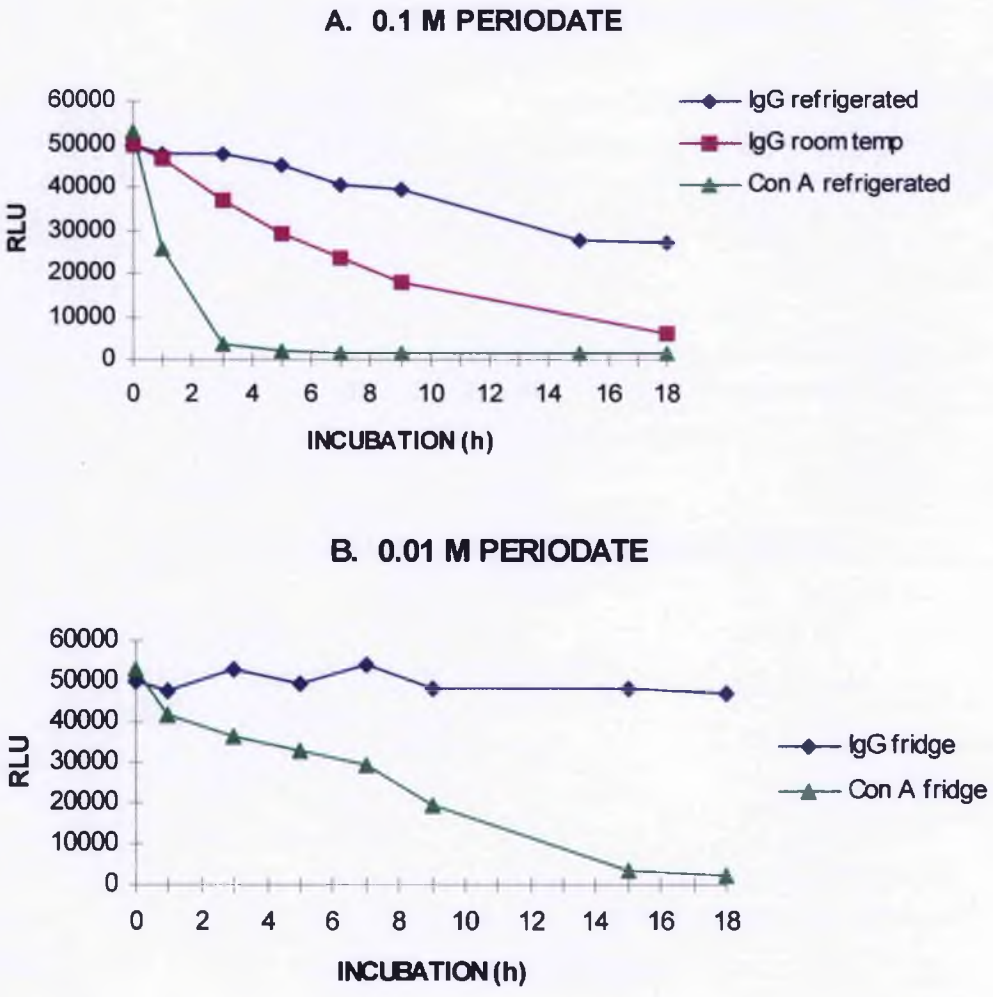


Figure 4.6. The effect on IgG measurement and Con A binding after treating capture antibody with (A) 0.1 M periodate and (B) 0.01 M periodate for different incubation periods. Incubation with periodate was at 4°C and also at room temperature with (A) 0.1 M periodate. Each point represents the mean of duplicate treatments. See 4.3.3.2 for method details.

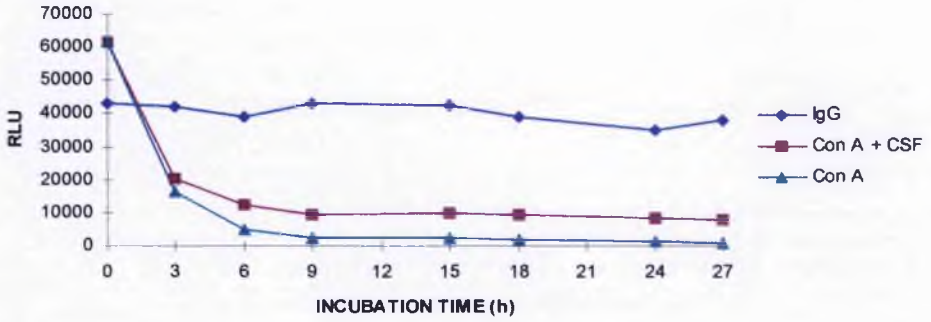


Figure 4.7. The effect of 0.01 M periodate at 4°C on capture antibody. Each point represents the mean of duplicate treatments. See 4.3.3.3 for method details.

Therefore to remove most carbohydrate on the capture antibody, whilst leaving the antibody still viable, 0.01 M periodate could be incubated with the coated tube at 4°C from 9 h to 27 h. For convenience, a 20 h incubation will be consistently used.

4.3.4.4. Results for Comparison of Periodate Treatments

Figure 4.8 compares the RLUs measured for a series of IgG calibration solutions assayed in antibody coated tubes treated by the three periodate protocols or not treated with periodate. As would be expected, antibody coated tubes not treated with periodate show highest binding of IgG. The different treatment protocols give similar results, although the 20 h treatment gives the lowest results. However removal of sugars is most effective using the 20 h treatment, as shown by Figure 4.9(C) which shows Con A binding directly to the capture antibody without addition of sample IgG. This is also seen in Figure 4.9(A), where CSF IgG is added and the RLU readings are a result of the CSF IgG sugars as well as those of the capture antibody. Figure 4.9(B) shows similar reductions in

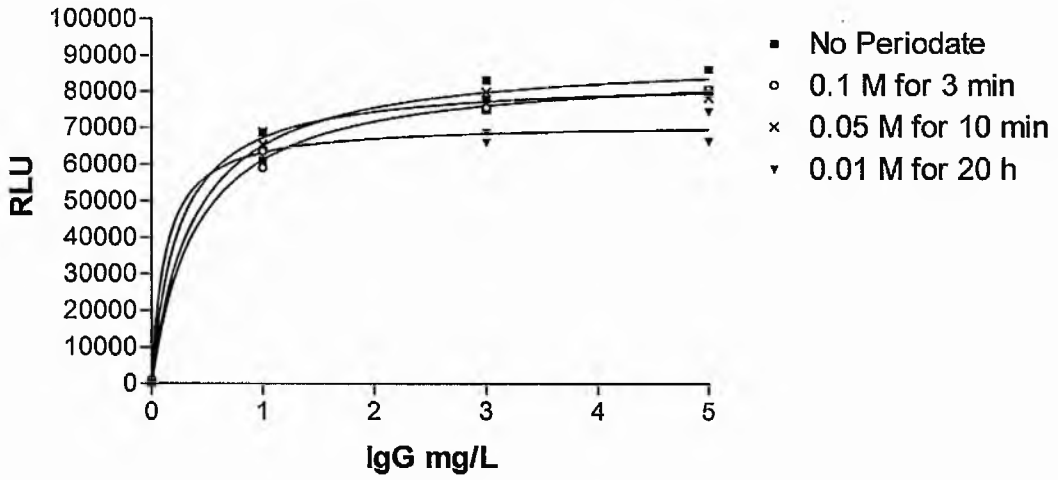


Figure 4.8. Assay of IgG standard solutions using antibody coated tubes deglycosylated by different periodate protocols or not treated with periodate. Duplicate points are shown for the standards in each assay. See 4.3.3.4 for method details.

RCA binding for the 3 periodate methods, suggesting that RCA-binding sugars in IgG are more easily oxidised than Con A binding sugars.

4.3.4.5. Results for Dilution of Capture Antibody

Figure 4.10 shows that the 1:100 dilution of capture antibody gives the highest binding of IgG. The shape of the curve is similar to that with 1:400 dilution of antibody without periodate treatment (Figure 4.1), while the other dilutions show plateauing of the curve at much lower levels.

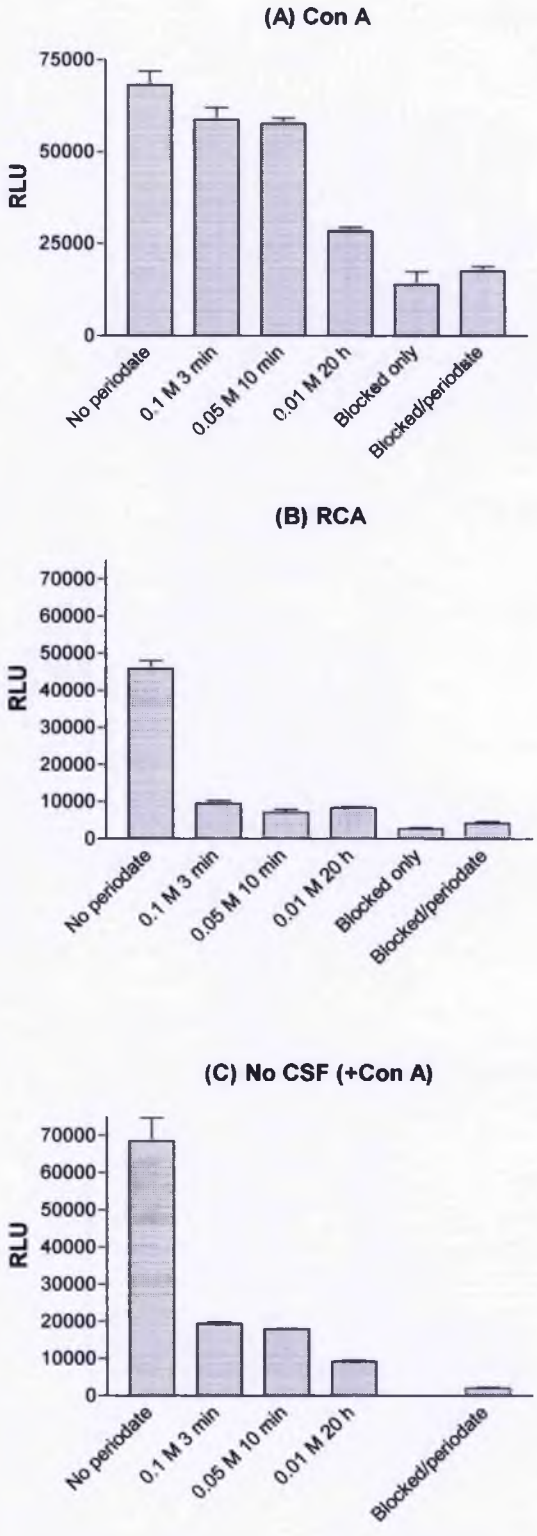


Figure 4.9. Comparison of 3 protocols for deglycosylation of capture antibody by treatment with periodate. (A) and (B) show binding of Con A and RCA respectively to capture antibody and CSF IgG after addition of CSF. (C) shows Con A binding with no addition of CSF. Error bars represent SEM of triplicate samples. See 4.3.3.4 for method details.

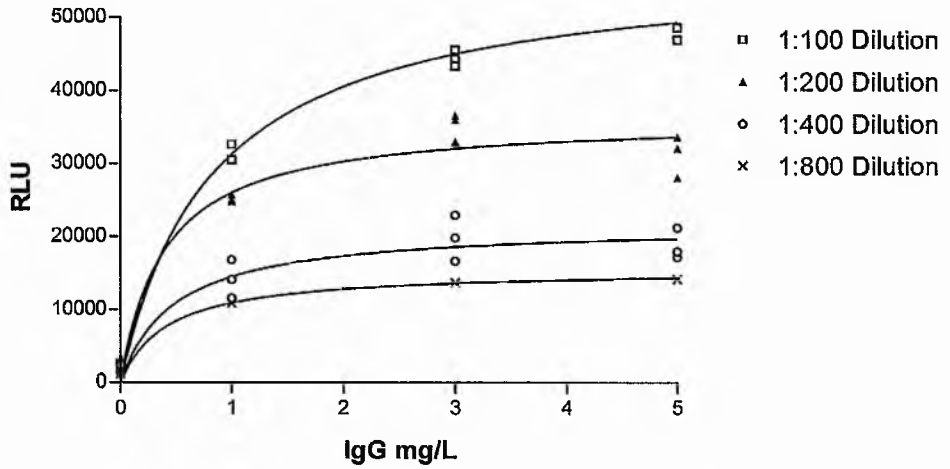


Figure 4.10. IgG binding with varying dilutions of capture antibody treated with 0.01 M periodate for 20 h. IgG standards were sampled in triplicate in each assay. See 4.3.3.5 for method details.

4.3.4.6. Results for Use of Ethanolamine and a Con A Inhibitor

Figure 4.11 shows the Con A binding assays of two CSF samples when the assay tubes are prepared in different ways. Antibody coated and blocked tubes (i.e. 'Coat/block/ethanolamine' and 'coat/block/no ethanolamine') give high RLUs for assay of both CSFs, and are similar whether or not the tubes were incubated with ethanolamine. However, tubes that were blocked without an antibody coat (i.e. 'no coat/block') show a substantial amount of Con A binding. The tubes without sample added (i.e. 'No sample') have low counts compared to tubes with CSF, showing that the Con A is itself not binding to the tube, antibody coat, nor blocker. Therefore there must be some substance (or substances) in CSF which is glycosylated and which binds avidly to the tube, coating antibody, blocker, or captured IgG (all or any of these) to which Con A is then binding. The inhibitor drastically reduces the binding of Con A in the assay, indicating that the lectin is not binding non-specifically to any great extent, but is binding to carbohydrates in this substance. The only slightly lower counts obtained for uncoated tubes with inhibitor suggest there is negligible Con A binding to the coating antibody.

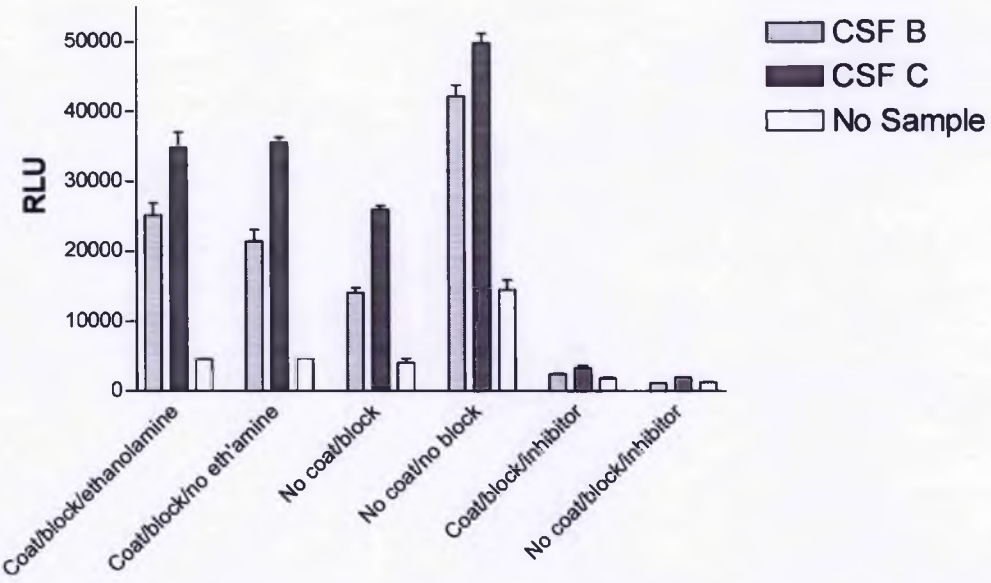


Figure 4.11. Comparison of assay tubes prepared in various ways, incubating with CSFs B, C or with no CSF and binding to Con A. Error bars represent SEM of duplicate samples. See 4.3.3.6 for method details.

4.3.4.7. Results for Analysis of IgG Glycosylation Using a Panel of Lectins.

a. IgG Assay

The results for the IgG concentrations of the four CSFs agreed fairly well with the results by IT (Table 4.2). The mean RLUs for the CSFs diluted to contain 10 mg/L of IgG (the dilution used for the lectin assays) are also shown. These are well above the mean RLU of the highest IgG calibrator (5 mg/L), indicating that, in the lectin assays, the capture antibody, with each CSF, should be fully - or almost fully - saturated with sample IgG.

b. Lectin Immunoassay

Figure 4.12 shows the RLUs obtained for each CSF for all 15 lectin assays. The chart 'No CSF' shows the lectins' binding to the capture antibody without sample IgG.

Table 4.2. Results of the IgG assay of the four CSFs used for the immunoassays with a panel of lectins.

CSF	IgG mg/L by IT	IgG mg/L by LIA	Mean RLU (n=2) for dilution containing 10 mg/L IgG.
A	22	16	51674
B	72	91	51140
C	25	26	52588
D	17	19	54797

On each chart is shown the results using antibody coated, blocked tubes (grey columns) and results using blocked only tubes, without an antibody coat (black columns). For each of the four CSFs it can be seen that lectin binding to the blocked-only tubes is considerable for most of the lectins. The lectins are not binding directly to the blocker, as can be seen in the chart 'No CSF' in Figure 4.12, where binding for 'no coat' tubes (black columns) is relatively small. Again it would appear that there are glycosylated substances in CSF which bind to the blocking agent and coating antibody, to which the lectins are binding.

The 'No CSF' chart in Figure 4.12 also shows that binding to the capture antibody is minimal for most lectins, with some exceptions. These will be discussed in the next section.

Figure 4.13 shows the same data as Figure 4.12, but the RLUs for the uncoated tubes have been subtracted from the antibody coated tubes. This is an attempt to correct for the binding due to the interfering substance, but is probably grossly inaccurate, as will be discussed in the next section.

4.3.5. Discussion for Deglycosylation of Capture Antibody

Incubation with 0.01 M periodate at 4° C for up to 27 h was shown to deglycosylate the capture antibody so that subsequent binding to Con A was minimal while the activity of the antibody was largely preserved. This method was found to deglycosylate the antibody more thoroughly, as shown by Con A binding, than the two other methods of periodate treatment. The 3 minute method is thought not to be suitable, as timing would be too critical when treating a large number of tubes. The 10 minute method (with 0.05 M periodate) could be used, however, according to the literature (Marshall and Neuberger, 1972), the lower the periodate concentration, the less problems there are from secondary oxidation damaging the peptide, so the 20 h incubation method (with 0.01 M periodate) will be used henceforth.

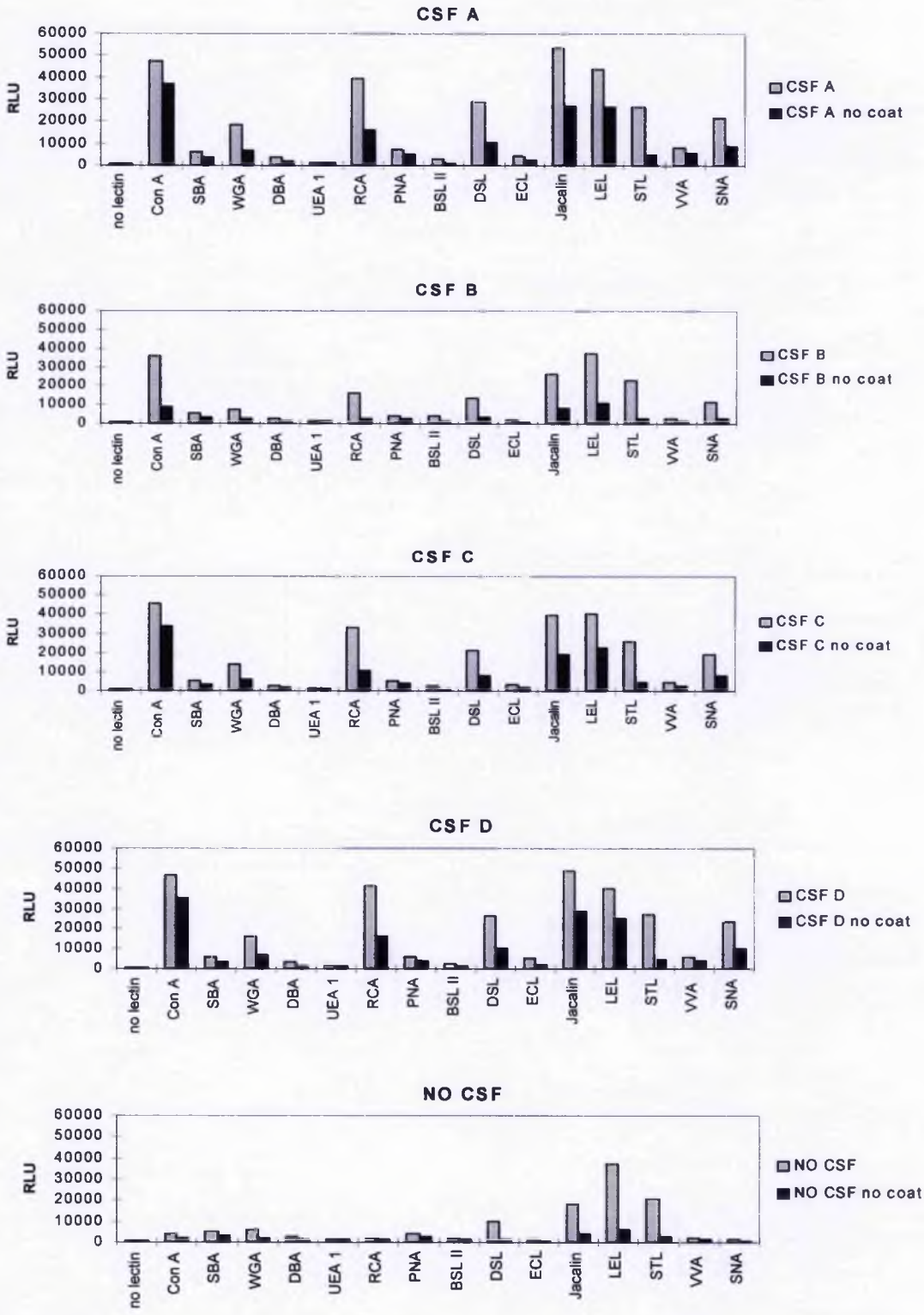


Figure 4.12. Binding of CSFs A, B, C and D in immunoassays using a range of lectins. The 'No CSF' chart shows lectin binding without addition of CSF sample. Columns are the mean of duplicate samples. Grey columns show results using antibody-coated and blocked assay tubes; black columns show results using blocked-only tubes. See 4.3.3.7(b) for further method details.

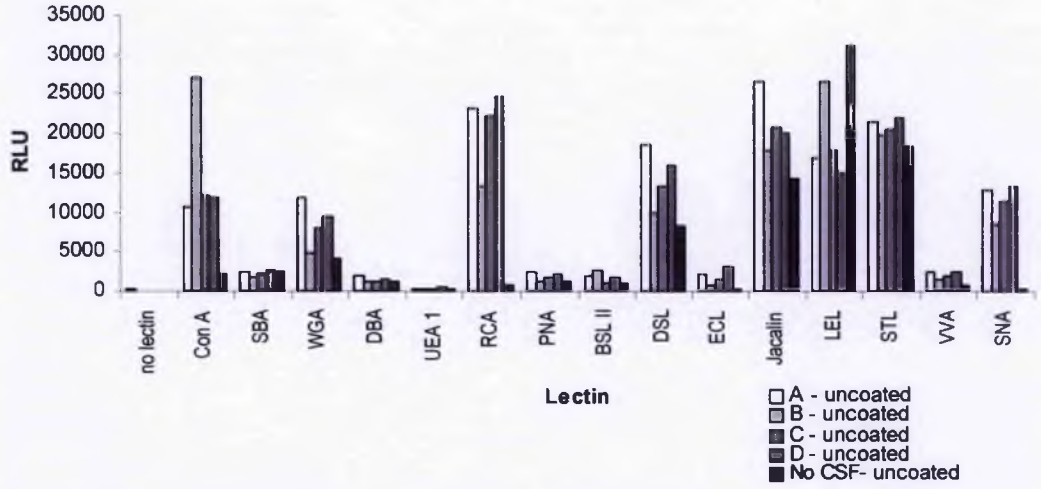


Figure 4.13. Subtraction of RLUs for blocked-only tubes from RLUs for antibody-coated tubes for the four CSFs and No CSF assay (Figure 4.12). Each column is the mean of duplicate samples. See 4.3.3.7(b) for method details.

It was eventually concluded that 1:100 dilution of the capture antibody was better than 1:400 with the 20 h periodate treatment, and although 1:400 was used in most of the studies described here, for the final lectin assays, described in section 4.6, 1:100 dilution was used.

Binding of most lectins to the deglycosylated capture antibody is shown to be minimal (Figure 4.12: 'No CSF') with some exceptions, notably WGA, DSL, jacalin, LEL and STL. This is probably due to incomplete glycosylation of the antibody. WGA, DSL, LEL and STL are known to bind to GlcNAc monomers or dimers. The first two sugars in an *N*-linked glycan are both GlcNAcs, and it is probable that the first one, or possibly both, are not completely destroyed by the periodate treatment. Jacalin, however, does not have an affinity for GlcNAc, but preferentially binds Galβ1-3GalNAc, Galβ-, and NeuAc2- linked to -3Gal and 3GalNAc, which are not found in IgG. The only human immunoglobulin that jacalin is said to bind to is IgA, so this suggests that IgA might be a component of the anti-IgG antibody.

Despite deglycosylation of the capture antibody, there is still a large and variable amount of background binding by the lectins in the immunoassays. This was effectively abolished for Con A by incubation with the inhibitor α-methylpyrannoside, indicating that the lectin is binding in a specific way to carbohydrate. Also it had been thought that the periodate oxidation of the antibody carbohydrates was producing reactive groups such as aldehydes which could be binding biotin, or the isoluminol-streptavidin and thus producing the high background binding. However, incubation of the periodate-treated antibody with ethanolamine, a primary amine which would neutralise such active groups, did not lead to a reduction in the background binding of Con A.

So it would appear that there is a substance (or substances) in CSF which is glycosylated and which binds avidly to the antibody coated tube and the blocking agent, and the lectins are binding to the sugars present in this. The same thing occurs with serum in lectin immunoassays, as found by Dr G. Firth while working on lectin immunoassays for serum transferrin (data not published). The phenomenon is not reported in several papers describing lectin immunoassays of IgG without prior purification of the IgG (Casburn-Budd et al, 1992; Keusch et al, 1996; Köttgen et al, 1993). None of these give any data for non-antibody-coated control tubes, so it is assumed that control tubes were not run.

With most lectin immunoassays, however, the analyte is purified before the immunoassay stage by affinity or ion-exchange chromatography. Similarly, with this project, some means must be found of removing, or possibly compensating for, this interfering substance. The feasibility of subtracting the measured RLUs from blocked-only control tubes from coated tube for every sample assayed has been considered (Figure 4.13). However, the amount of lectin binding to the control tubes is not necessarily the same as, or even in proportion to, the background binding of the lectin to the antibody-coated tubes. For example, the amount of IgG in the sample CSF would reduce

interferant binding by blocking sites on the capture antibody. So using blocked-only control tubes as sample blanks is not really viable. The nature of the interferent and the ways of removing it will be investigated in the next section. It would be expedient to actually identify the substance(s), but this is probably beyond the scope of this project.

4.4. Investigation of the Interferent

4.4.1. Introduction

The nature of the interferent was investigated and various ways of removing it, or compensating for it, were tried. These included dilution of CSF so that the IgG added to the assay tube was just saturating the capture antibody, but the interferent was so dilute as to be ineffective. Also polypropylene tubes were antibody coated for the assay, as it was thought the interferent might bind less to this material. Storage of CSF in glass containers was investigated as it was thought the interferent bound more to this material and so it might be a means of removing it. Extraction of IgG from CSF was tried using ion-exchange particles (DEAE), salt fractionation (with Rivanol), and Protein A. (The use of Protein A will be given in section 4.5.) Not all the data obtained will be presented here, as much was inconclusive and because of the need to reduce the size of the thesis. Similarly, attempts to automate the tube coating, and other assay steps, on the LIA analyser will not be shown.

4.4.2. Reagents

Reagents were as already given with the following substitutions or additions:

1. Coating buffer. Carbonate-bicarbonate buffer capsule: Sigma C-3041. The contents of one capsule were dissolved in 100 mL deionized water to produce a 0.05 M carbonate-bicarbonate buffer, pH 9.6.
2. Ethanolamine. From Merck as a 10% (^{v}/v) aqueous solution. 1% (^{v}/v) (0.16 M) and 0.2% (^{v}/v) (0.03 M) solutions in PBS were prepared.

3. DEAE. Sephacel, wet particle size 40 to 150 μm, product no. 17-0500-01, Pharmacia Fine Chemicals AB, Uppsala, Sweden.
4. Equilibration/elution buffer, 0.02 M, pH 7.2.

   A = Na_2HPO_4 (0.1 M; 14.2 g/L); B = KH_2PO_4 (0.1 M; 13.6 g/L)

   7 mL of A was mixed with 3 mL of B, diluted with 40 mL of water and adjusted to pH 7.2.
5. Final buffer. 1 M KCl in equilibration/elution buffer.
6. Coomassie blue CSF total protein assay was performed by the method of Cowdrey (1991) using a Cobas-Bio centrifugal analyser (Roche Diagnostics).

4.4.3. Methods

All washing steps consisted of 3 additions of 1.5 mL of saline-Tween, with aspiration after each addition. All incubations were at room temperature.

4.4.3.1. Serial Dilution of CSFs: Assay with Con A, RCA and anti-IgG

200 assay tubes were coated overnight with 0.2 mL anti-IgG diluted 1:400, washed then blocked with 0.3 mL of 10 g/L BSA for 1 h. 100 plain tubes were also blocked in a similar way. 0.4 mL of 0.01 M periodate in citrate buffer was added to all tubes apart from 50 coated and 50 uncoated and incubated at 4°C for 20 h. 0.4 mL of 1% (^{v}/v) ethanolamine and 0.4 mL of 0.2% (^{v}/v) ethanolamine was added to 64 coated and 64 uncoated tubes each and incubated for 2 h.

CSF A, oligoclonal IgG negative, and CSF B, oligoclonal IgG positive, both coincidentally with an IgG of 46 mg/L by IT, were each diluted with PBS-Tween to 10 mg/L IgG. Human IgG (from Sigma) was diluted to 10 mg/L. The three solutions were then diluted with PBS-Tween to give a range of IgG concentrations for each as described in section 3.2.3.1.

0.4 mL of all IgG solutions was diluted with 8 mL PBS-Tween and immediately 0.2 mL of this was added to labelled assay tubes in duplicate and incubated for 2 h, then

washed. Biotinylated lectins were diluted 1 in 1000 with lectin assay buffer, and 0.3 mL added to relevant tubes and incubated for 1.5 h, then washed. 0.2 mL of streptavidin-isoluminol diluted 1:1000 with PBS-Tween was added to each tube, incubated for 45 min and washed and tubes counted. For the IgG assay, 0.2 mL of anti-IgG-ABEI diluted 1:1000 with PBS-Tween was added to the relevant tubes, incubated for 1.5 h, washed and counted.

4.4.3.2. DEAE Column Fractionation of CSF

The DEAE resin was prepared by equilibration over 4 h periods in 3 changes of Tris-saline. A column of 5 cm length was prepared, with a capacity of 1.2 mL.

a. First Fractionation

200 µL of a CSF sample ('BM' - with [IgG] of 78 mg/L) was applied to the top of the column and 200 µL amounts of Tris-saline were applied to the column and the eluate collected in a glass vial after each application. Nine fractions were collected. The fractions were assayed for (i) total protein using Coomassie blue and (ii) IgG by IT, both methods following Cowdrey, 1991.

As described in section 4.3.3.6, 20 assay tubes were anti-IgG coated and periodate treated, then blocked along with 20 plain tubes. CSF BM was diluted 1:20, 1:40, 1:80 and 1:160 with saline. 200 µL of assay buffer, followed by 10 µL of CSF, diluted CSF or column fraction was pipetted into labelled tubes in duplicate and incubated for 1.5 h. After washing, 200 µL of Con A-biotin, diluted 1:10000 with lectin assay buffer was added and incubated for 1.5 h. After washing, 200 µL of isoluminol-streptavidin diluted 1:10 000 was added and incubated for 1 h, washed and RLUs measured.

b. Second Fractionations

Two samples, CSF H (total protein = 451 mg/L; IgG = 56 mg/L; oligoclonal IgG bands positive) and CSF W (total protein = 188 mg/L; IgG = 13 mg/L; oligoclonal IgG

negative) were processed as follows. 200 µL of CSF was applied to the column, followed by 200 µL amounts of equilibration/elution buffer, with collection of eluted fraction. Ten such fractions were collected. Then 200 µL amounts of final buffer were applied to the column, and 8 more fractions were collected. The fractions were assayed for total protein by Coomassie blue and IgG by IT.

Fractions 3, 4 and 5, containing IgG, were pooled and along with the untreated CSFs, portions were diluted to contain 5 mg/L of IgG, and then assayed with Con A as described in (*a*) above, but with sampling in triplicate rather than duplicate. Fractions 6 to 18 were also pooled and, with the IgG pools and the untreated CSF, were assayed for IgG according to the protocol in section 3.3.3.1 after suitable dilution (1:13 for pools 3 to 5; neat for pools 6 to 18 and 1:40 for untreated CSFs).

4.4.3.3. Effect of Storage at 4°C on IgG and Interferent

For the IgG assay, 50 tubes were coated overnight with 200 µL of anti-IgG diluted 1:400, washed and blocked with 300 µL of 10 g/L BSA. IgG calibrators were prepared as described in section 3.2.3.1. For Con A assays, 100 tubes were antibody coated, blocked and treated with periodate, and 100 tubes were blocked only, as described in section 4.3.3.6.

Tubes were coated with different amounts of human IgG (from Sigma), to compare with the tubes in which the CSF had been stored. Thus, 10 µL of IgG was diluted with 4 mL PBS; of this, aliquots of 50, 100, 150 or 200 µL, containing respectively 0.7, 1.4, 2.1 and 2.8 µg of IgG, were placed into four assay tubes each and incubated overnight, then washed.

Three CSF samples, Y, W and E, with IgG levels by IT of 60, 50 and 43 mg/L respectively, were obtained on the day of the lumbar puncture. 100 µL aliquots of each CSF were placed into glass bottles and stored at -70°C, while 100 µL aliquots were placed

into 4 polystyrene tubes for each CSF and placed at 4°C along with the remaining samples in the collection tubes. For CSF Y, 100 μL was placed in four additional polystyrene tubes and stored at -70°C. Thereafter, at weekly intervals for 4 weeks, 100 μL from each CSF collection tube was placed into glass bottles and stored at -70°C.

All frozen samples were thawed at room temperature just before use. For the IgG assay, all samples were diluted 1:40 and assayed as described in section 4.3.3.6. For the Con A assays, all CSFs were used neat and also diluted to contain 5 mg/L IgG. 200 μL of PBS-T followed by 10 μL of sample was pipetted into periodate-treated coated tubes in duplicate and corresponding blocked-only control tubes, incubated for 2 h and washed. 200 μL of biotinylated Con A diluted 1:1000 with lectin assay buffer was added to all tubes and incubated for 1.5 h then washed. 200 μL of isoluminol-streptavidin diluted 1:1000 was added to all tubes, incubated for 1 h, washed and measured.

The polystyrene tubes used for storing the CSFs at 4°C for 4 weeks, and those containing CSF Y kept at -70°C, were washed x3 and assayed along with the IgG coated tubes. For IgG measurement, diluted anti-IgG-ABEI was added directly to the tubes and the assay continued as described above for the IgG assay. For the Con A assay, diluted biotinylated Con A was added directly to the tubes, and the assay continued as described above for the Con A assay.

4.4.4. Results

4.4.4.1. Results for Serial Dilution of CSF: Assay with Anti-IgG, Con A and RCA

Graphs showing anti-IgG binding to dilution curves of purified IgG, CSF A and CSF B are shown in Figures 4.14(a), (b) and (c) respectively. The curves are of similar shape for all three samples, and reach a plateau by 5 mg/L IgG, indicating near saturation of the capture antibody at this level. In the lectin assays, dilution of samples to 5 mg/L

IgG would ensure that the capture antibody is saturated and enable comparison of results of different samples for a particular lectin.

In all three graphs, periodate treated tubes show less binding capacity than non periodate treated tubes, indicating the capture antibody is more damaged by the periodation method than was previously suspected, when over 95% of the activity was seen. The damage to the antibody appears to have a variable effect on its binding to the IgG in different samples: with CSF A, Figure 4.14(b), there is 55% of the original activity; with CSF B, Figure 4.14(c), there is 90% activity. Treatment of coated tubes with ethanolamine reduces IgG binding, with the effect of 1% (^{v}/v) solution more than 0.2% (^{v}/v).

Figure 4.15 shows binding of Con A and RCA for different dilutions of CSF A [(a) and (b) respectively] and for CSF B [(c) and (d) respectively]. The effect of ethanolamine on CSF A with Con A and RCA is shown in (e) and (f) respectively.

Graphs (a) to (d) for antibody coated tubes (open square symbol) show that there is increasing binding of both lectins even beyond the point where the capture antibody is largely saturated (i.e. 5 mg/L IgG). The increasing binding must be attributable to increasing binding of the interferent, as suggested by the non-antibody-coated tubes (filled squares). The graphs for coated and uncoated tubes are linear apart from Graph (a) where there is a flattening of the graph. Unless the interferent is eliminated, the chosen saturating concentration of the CSF IgG would be critical in the lectin assays. Dilution of CSFs to contain 5 mg/L IgG has been considered, but a level of 10 mg/L might produce very different data and a different conclusion.

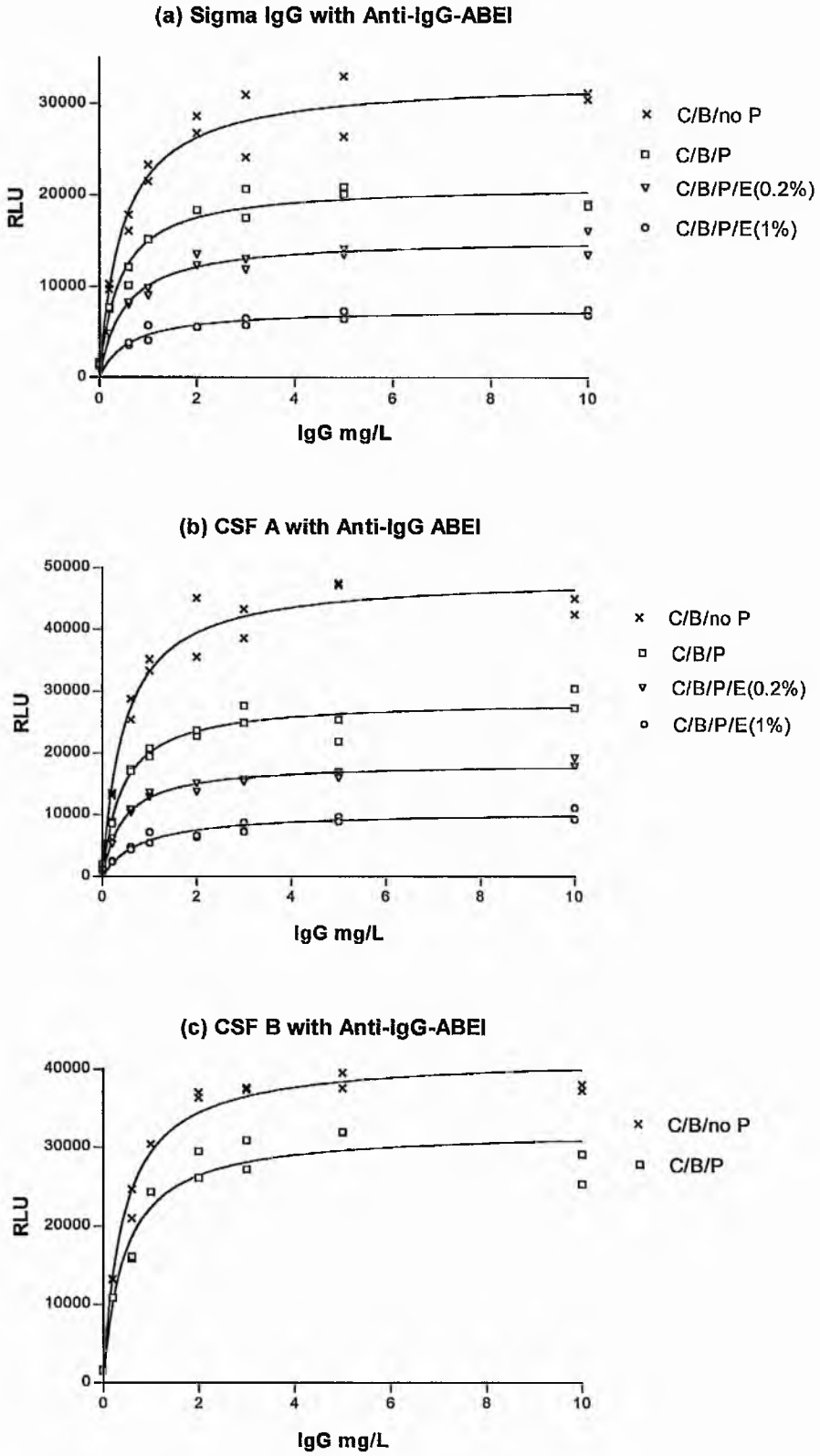


Figure 4.14. Serial dilutions of (a) purified IgG, (b) CSF A and (c) CSF B assayed with Anti-IgG-ABEI. Legends indicate the following preparations to assay tubes: C = coated with anti-IgG; B = blocked with 10 g/L BSA; P = periodate treated; no P = not periodate treated; E = treated with 0.2% (^{v}/v) or 1.0% (^{v}/v) ethanolamine. Points for samples assayed in duplicate are shown. For further experimental details, see 4.4.3.1.

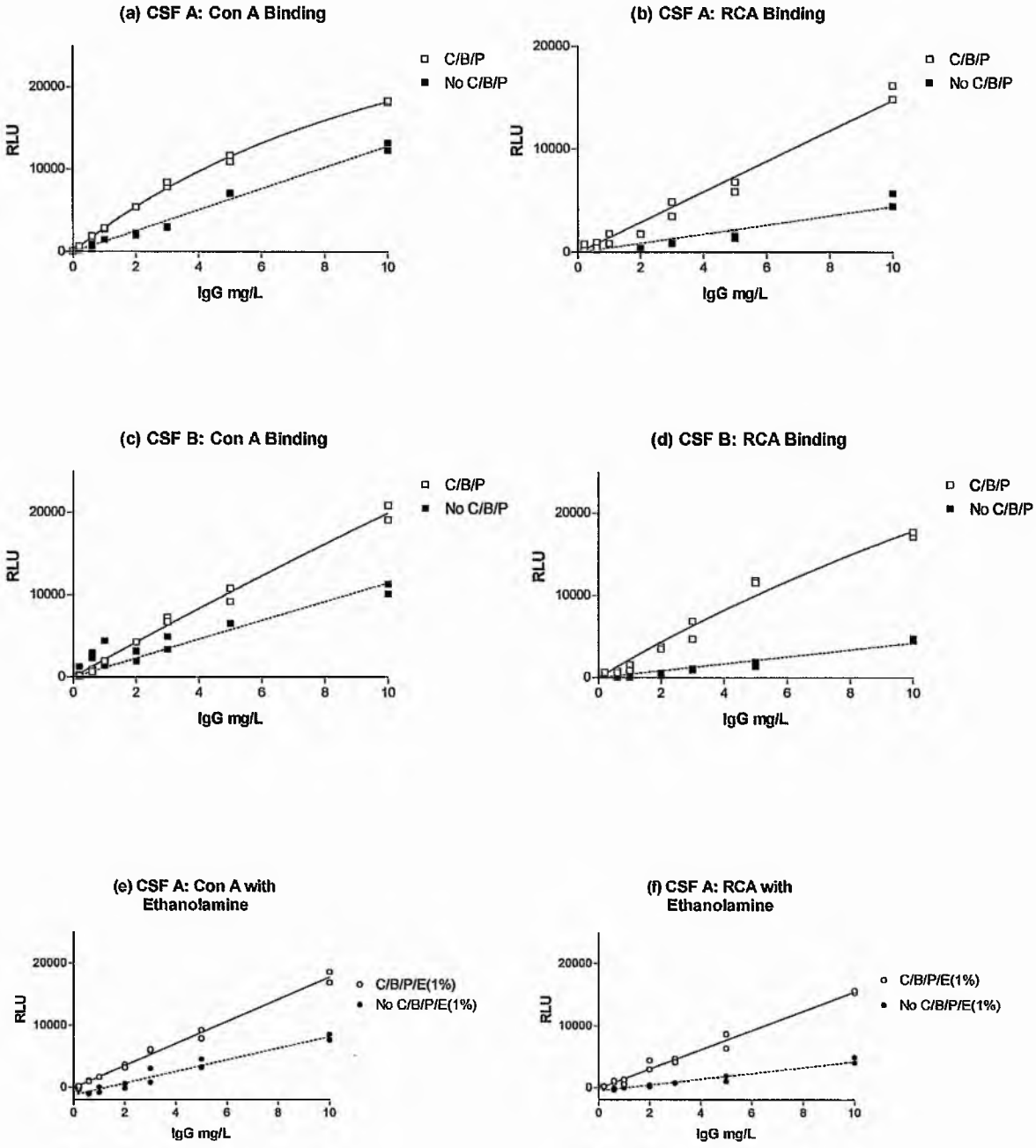


Figure 4.15. Serial dilutions of CSF A and CSF B assayed with Con A or RCA. Legends indicate the following preparations to assay tubes: C = coated with anti-IgG; B = blocked with 10 g/L BSA; P = periodate treated; no P = not periodate treated; E = treated with 1.0% ($^{V}/v$) ethanolamine. Points for samples assayed in duplicate are shown. For further experimental details see 4.4.3.1.

Graphs (e) and (f) show that ethanolamine does not have an effect on binding of either lectin with coated or uncoated tubes. Generally the patterns of the graphs are the same as for graphs (a) and (b), although there is some degree of imprecision at the low IgG levels, and in some cases the RLUs for 0.2 and 0.6 mg/L IgG were lower than RLUs for the 0 mg/L IgG, and so the regression lines do not pass through the origin.

4.4.4.2. Results for DEAE Column Fractionation of CSF

a. Results for First Fractionation

Figure 4.16 shows the IgG and total protein concentrations in the DEAE column fractions of CSF BM. IgG is seen to elute in fractions 3, 4 and 5. Figure 4.17 shows the Con A binding to CSF BM, to a range of dilutions of BM and to the DEAE fractions 3 and 4 of BM with both antibody coated tubes and blocked only control tubes. In the neat CSF, interferent binding, reflected by the control tubes, is very large and must contribute considerately to the Con A binding with the antibody coated tubes. With 1:20 dilution of the CSF, the interferent as well as the IgG is diluted and overall Con A binding with coated tubes is reduced. With further dilution, the interferent binding in the control tubes decreases, but binding in the antibody coated tubes remains fairly constant. This indicates that more IgG can bind to the antibody after removal of the interferent. Fractions 3 and 4 do not appear to have much advantage in terms of reduction of interferent compared to

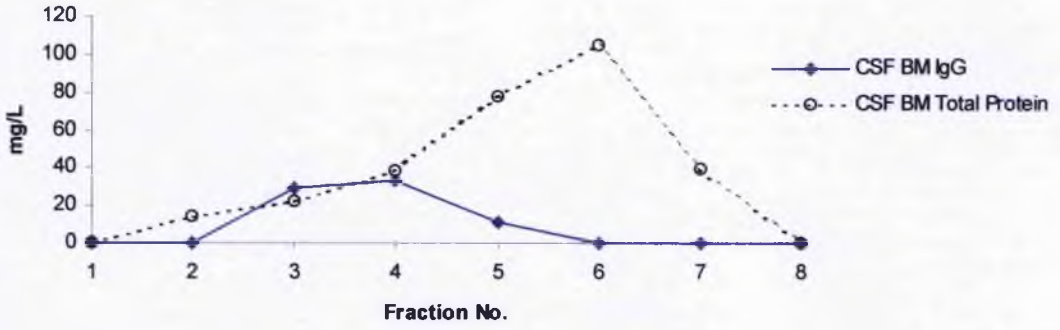


Figure 4.16. Measured IgG and total protein in DEAE column fractions of CSF BM. For further details of method see 4.4.3.2.

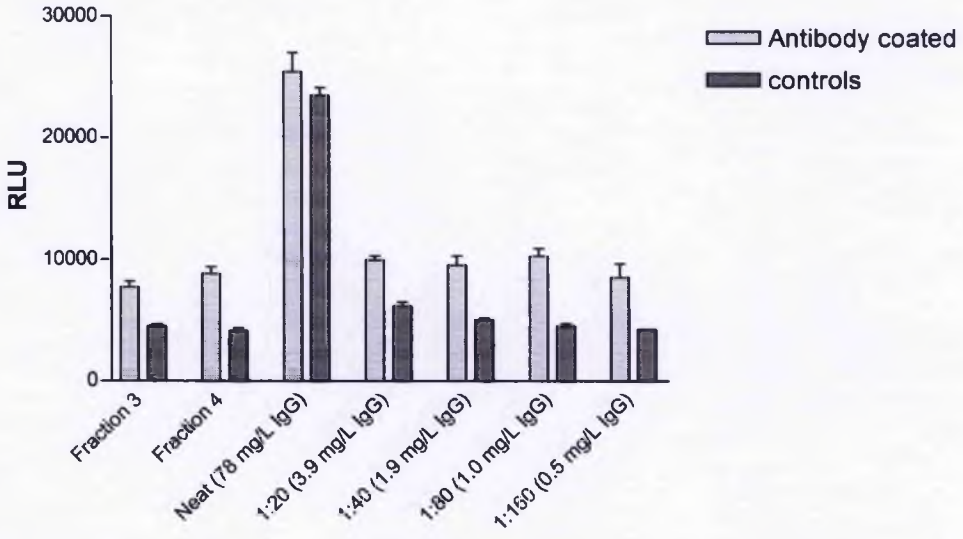


Figure 4.17. Con A immunoassay, using antibody coated tubes and control (blocked only) tubes, of CSF BM, a range of dilutions of CSF BM, and DEAE column fractions of CSF BM. Error bars represent SEM of duplicate samples in the immunoassay. For further method details see 4.4.3.2(a).

the diluted CSF. Unfortunately a 'no sample' blank was not run, so the background Con A binding cannot be assessed.

b. Results for Second Fractionations

The IgG and total protein measurements, expressed as absorbance changes for the DEAE column fractions for CSFs H and W are shown in Figures 4.18(a) and (b) respectively. Again there is a definite IgG peak in fractions 3, 4 (and 5), with very little IgG subsequently eluted even after addition of KCl. The Con A binding of the pooled fractions 3, 4 and 5 compared with the whole CSFs, after dilution to 5 mg/L IgG, is given in Figure 4.19. With dilution of the CSFs, the interferent binding (shown by the dark columns) is fairly small relative to the overall binding in the antibody-coated tubes (light columns). The interferent binding in the pooled fractions is slightly reduced compared to that in the whole CSFs.

The IgG measured in the pooled fractions 3 to 5 for CSFs H and W was used to calculate the recovery of IgG from the column. These were found to be only 34% and 18% respectively.

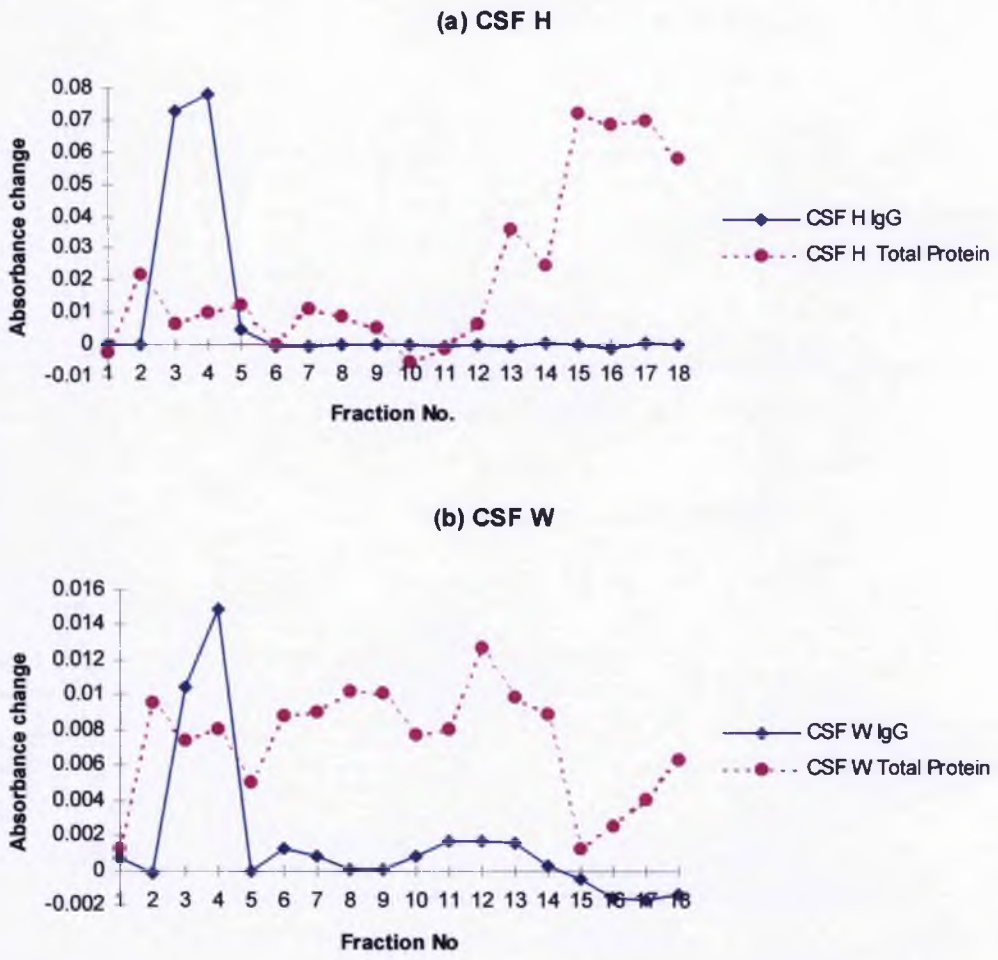


Figure 4.18. Absorbances of IgG and total protein assays of DEAE column fractions of (a) CSF H and (b) CSF W. KCl buffer was added to the column after collection of fraction 10. For further experimental details see 4.4.3.2(b).

4.4.4.3. Results for Effect of Storage at 4°C on IgG and Interferent

Figure 4.20 shows the IgG measured in (a) CSF Y, (b) CSF W and (c) CSF E after storage at 4°C for up to 4 weeks. There does not appear to be any decrease in IgG over this time. An odd result was obtained for CSF Y after 4 weeks [Graph (a)] where the IgG appears to increase compared to the other aliquots. It is possible that this was due to the freezing and thawing of the other aliquots prior to the assay, whereas the 4 week samples were not frozen as the assay was performed at 4 weeks from the beginning of the storage. However, this effect is not seen in the other two CSFs treated in the same way.

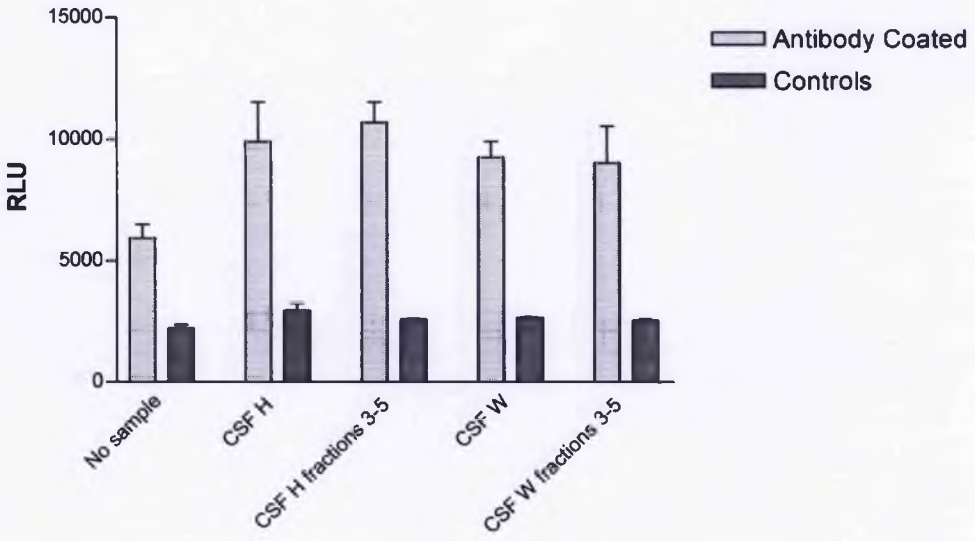


Figure 4.19. Con A binding to CSFs H and W and their pooled DEAE IgG fractions after dilution of all samples to 5 mg/L IgG. Error bars represent SEM of triplicate sampling for the immunoassay. For further experimental details see 4.4.3.2(b).

Figure 4.21 shows Con A binding to the three CSFs stored for different lengths of time. The charts for each CSF show the binding for blocked only control tubes as well as the standard anti-IgG coated, blocked and periodate treated tubes, and for CSF assayed neat and diluted to 5 mg/L. There is no evidence of a decrease in Con A binding with time due to removal of either IgG or interferent from the sample, except possibly for CSF E [chart (c)] where the neat CSF shows a downwards trend. However, this is not reflected in the controls or the dilution for CSF E and is probably due to assay imprecision.

The assay of the CSF storage tubes is shown in Figure 4.22. The prepared IgG coated tubes show increasing RLU for IgG with increasing IgG coating but a decrease with the 200 μg coating. This decrease is possibly due to steric hindrance from excessive coating. The Con A binding is similar in all the prepared tubes. In the sample tubes, the IgG binding is small, but Con A binding is relatively high, suggesting substantial binding of the interferent to the tubes.

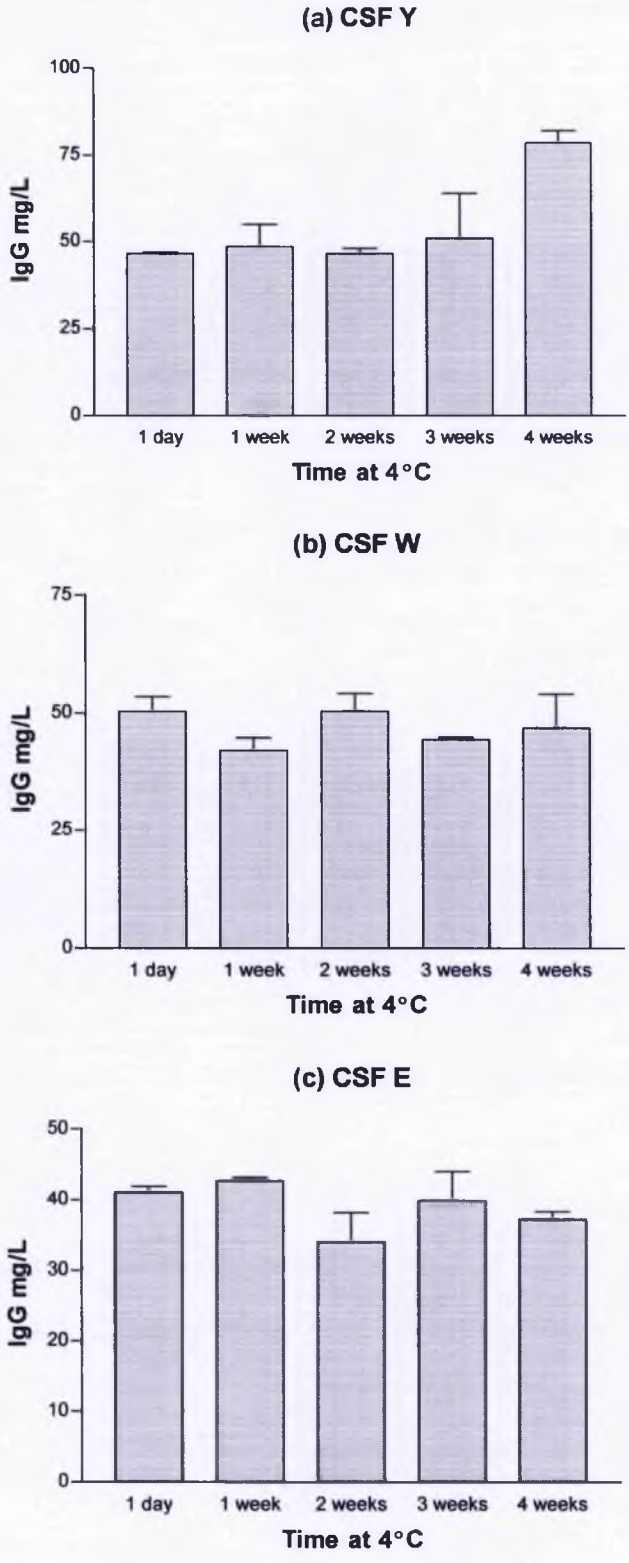


Figure 4.20. IgG measured in three CSF samples stored at 4°C for up to 4 weeks. Error bars represent SEM of duplicate sampling. See 4.4.3.3 for further experimental details.

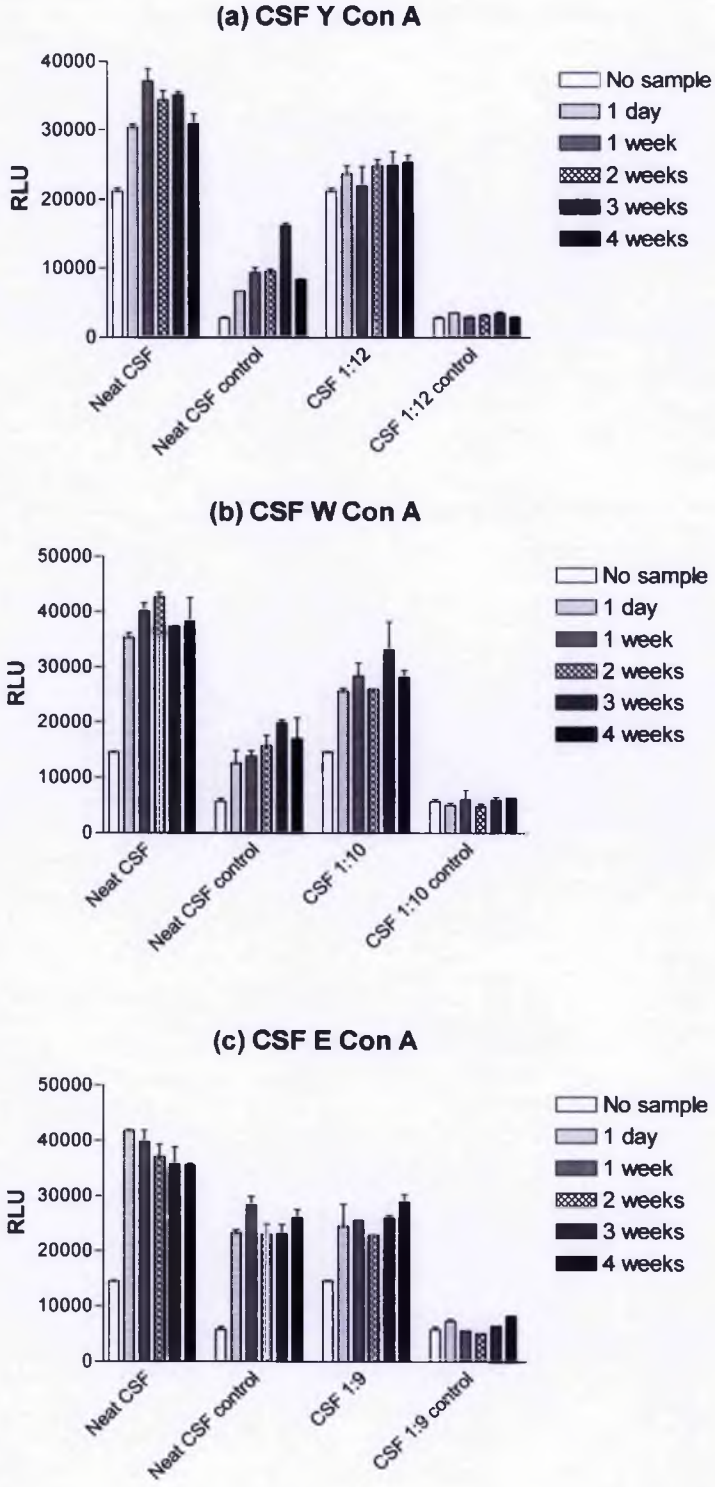


Figure 4.21. Con A binding for 3 CSFs after storage at 4°C for up to 4 weeks. CSFs were assayed neat and diluted to 5 mg/L of IgG. Control tubes are blocked only, with no antibody coating. Error bars represent SEM of duplicate samples. For further experimental details see 4.4.3.3.

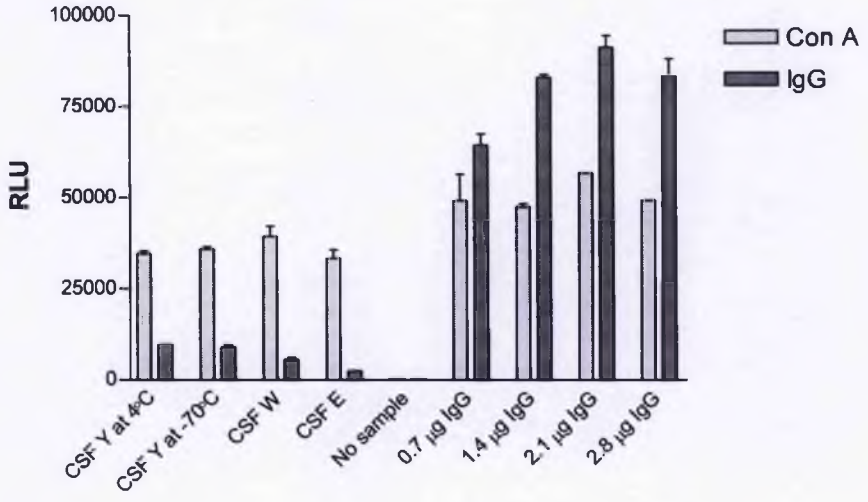


Figure 4.22. IgG and Con A assays of washed tubes used for storage of CSFs Y, W and E compared to a range of IgG coated tubes. Error bars represent SEM of duplicate tubes. For further experimental details see 4.4.3.3.

4.4.5. Discussion for Investigation of Interferent

The dilution studies have confirmed that using less CSF sample reduces the effect from the interferent. When the sample is diluted so that it contains 5 mg/L of IgG, the capture antibody is almost saturated with IgG, although absolute saturation is not reached even at the 10 mg/L level. The interferent causes much less lectin binding at 5 mg/L compared to 10 mg/L as judged by the blocked-only control tubes. So it seems expedient to dilute all samples to 5 mg/L for the lectin assays.

It is difficult to assess the effect of ethanolamine with the data obtained. With the IgG assays, ethanolamine added to periodate treated tubes caused a reduction in measured RLUs, suggesting that IgG may be binding to periodate-activated groups on the capture antibody. However, non periodate treated tubes gave higher binding with similar shaped graphs, so it would seem unlikely that non-specific IgG binding is caused by periodation [Figures 4.14(a) and (b)]. As the 1% (^{v}/v) ethanolamine treatment produces less binding than the 0.2% (^{v}/v), it is possible that ethanolamine treatment damages the antibody. Further work could include ethanolamine treatment of non-periodate treated tubes, to see

if it causes deterioration of binding. The lectin assays seem unaffected by ethanolamine treatment, so it does not appear to be the cause of the interference in the lectin assays, which was the subject of the experiment, so no further work was done.

The reduction of IgG binding to periodate treated tubes was greater than had been previously found and led to using 1:100 dilution of anti-IgG rather than 1:400 for tube coating.

With DEAE column fractionation of CSF, binding of the interferent was reduced, although simple dilution of the CSF produced almost the same reduction. However, with DEAE fractionation the recoveries of IgG were low, so more work would need to be carried out if DEAE purification were to be used for the lectin assays of patients' samples. A quantity of protein A (Prosep A) was donated, however, and affinity purification of samples with this is described in section 4.5.

The results showed that IgG was not reduced in CSF with storage at 4°C. However, assay of the storage tubes did show substantial Con A binding, so there may be adhesion of the interferent. Further work on storage of CSF will be described in the next section.

4.5. Protein A Separation of IgG

4.5.1. Introduction

Preliminary work (data not presented here) found that a 0.2 mL Prosep A column retained IgG better than a 0.1 mL column, and that there was little advantage using a stirred mode over a flow-through mode. This section will firstly describe the initial Con A assay of CSF fractions, followed by the selection of adsorption, elution and neutralising buffers. Then stepped elution studies are shown, measuring IgG, total protein and Con A binding (including blocked only control tubes to indicate interferent binding) with

assessment of IgG recovery. Finally, studies on the stability of IgG in the eluates under different storage conditions are described, together with ways to improve it.

4.5.2. Reagents

Reagents are as previously described, with the following additions.

1. Prosep A. Protein A immobilised on controlled pore glass. Bioprocessing Ltd., No 1 Industrial Estate, Consett, Durham, DH8 6JT. Kindly donated by Dr P. Kwasowski, Surrey University. 0.2 mL used in a 1.4 mL capacity polypropylene column.
2. Neutralising/Preserving buffer. 0.5 M phosphate buffer pH 7.4 containing azide. Dissolve (a) 15.3 g of $NaH_2PO_4.2H_2O$ and (b) 57.6 g of Na_2HPO_4 in about 950 mL of water. Add 5 g/L NaN_3. Test pH and adjust if necessary with small addition of (a) or (b), then make the volume up to 1 L with water.
3. Phosphate buffers pH 7.4, 0.5 M, 0.2 M and 0.1 M. Prepared from 0.5 M buffer.
4. Adsorption buffers:
    - NaCl 2 M. Dissolve 5.8 g NaCl in 50 mL PBS
    - Glycine 2 M, pH 7.0. Dissolve 7.5 g glycine in 50 mL PBS.
5. Elution buffers
    - HCl 0.03 M, pH 1.5. Dilute 0.14 mL concentrated HCl to 50 mL with water.
    - HCl added to water to give solutions of pH 2.0 and pH 2.5.
    - HCl/glycine pH 3.0. Dissolve 0.375 g of glycine with 0.292 g NaCl in 50 mL water. To 23 mL of this add 7 mL of 0.1 M HCl and adjust to pH 3.0.
    - Sodium citrate 0.5 M in water (pH 2.15).

- Disodium hydrogen phosphate (Na_2HPO_4) 0.5 M in water.
- Citrate/phosphate buffers: sodium citrate (0.5 M) + Na_2HPO_4 (0.5 M) mixed to give solutions of pH 2.8, 3.9, 4.9, 6.7 and 7.8
- Sodium citrate (0.5 M) + Na_2HPO_4 (0.5 M) with 2 M glycine (1.5 g/10 mL). Adjusted to pH 2.8.
- Potassium iodide 2 M (3.32 g/10 mL water)
- Sodium thiocyanate 2 M (5 g/10 mL water).

6. Anti human IgG conjugated with horseradish peroxidase. Product No PPO4, The Binding Site, Ltd. PO Box 4073, Birmingham B29 6AT.
7. o-Phenylenediamine dihydrochloride (OPD) and urea hydrogen peroxide tablets set. Sigma product No P-9187. The reagent was prepared just before use by dissolving one of each of the tablets in 20 mL water. This produces a 0.05 M phosphate-citrate buffer solution containing 0.4 μg/L OPD and 0.4 μg/L urea hydrogen peroxide.
8. Peroxidase stopping solution: 3 M HCl. Dilute 28 mL of conc. HCl to 100 mL with water.

4.5.3. Methods

4.5.3.1. Preparation of the Column

a. Initial Con A Assay of Protein A fractions

0.2 mL of Prosep A was placed in the column and, after draining, was re-suspended in 1.0 mL of PBS. After settling out, the buffer was aspirated, thus removing any fines, and flushed with 3 ml of 0.03 M HCl, followed by 3 mL of PBS.

0.2 mL of CSF P, containing 122 mg/L IgG, was applied to the column, followed by 0.2 mL of PBS with collection of the breakthrough (fraction P-1). 1.0 mL of PBS was applied to the column, followed by 0.2 mL of 0.03 M HCl, then another 0.2 mL of 0.03 M

HCl, collecting the eluate onto 0.2 mL of 0.2 M PBS (fraction P-HCl). After flushing the column with 1 mL of 0.03 M HCl and 1 mL of PBS, 1.0 mL of CSF B (containing 20 mg/L of IgG) was applied to the column and the breakthrough collected (fraction B-1). 1.0 mL of PBS was then applied to the column followed by 0.4 mL of 0.03 M HCl, collecting the eluate onto 0.4 mL of 0.2 M PBS (fraction B-HCl).

The collected fractions were assayed for IgG by IT and then, with the CSFs, were assayed with Con A after diluting the HCl fractions and the CSFs to 5 mg/L of IgG. The Con A assay was as follows:

Tubes were coated overnight with 0.2 mL of anti-IgG diluted 1:100, washed, then blocked for 1 h with 0.2 mL 10 g/L BSA, washed and treated with 0.3 mL of 0.01 M periodate for 20 h at 4°C. Control tubes were blocked only with 0.2 mL 10 g/L BSA. 200 µL of PBS-Tween was placed into prepared tubes followed by 10 µL of sample in triplicate and incubated 1.5 h. After washing, 0.2 mL of biotinylated lectin (diluted 1 in 1000 with lectin assay buffer) was added and incubated for 1.5 h. After washing 0.2 mL isoluminol-streptavidin was added (diluted 1 in 1000 with lectin assay buffer) and incubated for 45 min. After a final wash, RLUs were measured.

b. Adsorption Buffer

Three CSFs were pooled to give sufficient volume. The column was primed with 0.4 mL of the first buffer, then 0.2 mL of the buffer was mixed with 0.2 mL of CSF and applied to the column. A further 0.4 mL of the buffer was added. Then 0.1 mL of 0.03 M HCl was added, followed by a further 0.4 mL with collection of eluate onto 0.1 mL of 0.5 M phosphate buffer. The column was cleaned with a further 0.4 mL of 0.03 M HCl, then the procedure repeated with the next buffer.

c. Elution Buffer

2.5 mL of pooled CSF samples was mixed with 2.5 mL of PBS/glycine. 0.4 mL of the mixture was applied to the column, followed by 0.4 mL of PBS/glycine. 0.2 mL of

the first eluant was applied, followed by another 0.4 mL with collection of the eluate. The column was cleaned with 0.4 mL of 0.03 M HCl then equilibrated with 0.4 mL of PBS/glycine and the procedure repeated with each eluant in turn.

d. Column Capacity

The column was primed with 1.0 mL of PBS, then 14 x 100 µL aliquots of CSF (IgG measured by IT was 76 mg/L) were applied to the column and the breakthrough collected after each addition. IgG (IT) was then measured on each breakthrough fraction.

e. Neutralizing Buffer

The selected elution buffer was mixed in the ratios 1:1 with 0.01 M PBS pH 7.6, 0.1 M, 0.2 M, and 0.5 M phosphate buffer pH 7.4, and also in the ratios 2:1, 3:1 and 4:1 with the 0.5 M phosphate buffer. The pH of the mixtures was measured with pH paper and confirmed with a pH meter.

4.5.3.2. Stepped Elution Studies

a. Stepped Elution with Buffers of Decreasing pH

0.4 mL of pooled CSF ([IgG] = 56 mg/L) was mixed with 0.4 mL of adsorption buffer (PBS containing 2 M glycine) and applied to the column and the breakthrough collected as Fraction 1. Then 0.4 mL of adsorption buffer was applied, collecting the breakthrough as Fraction 2. Then in turn, 0.4 mL aliquots of buffers of pH 7.8, 6.7, 4.9, 2.8 and 1.6 were applied to the column, collecting the eluates as Fractions 3 to 7. 0.1 mL of neutralising/preserving buffer was added to each fraction bottle prior to collection.

The fractions were then assayed for total protein by Coomassie Blue (Cowdrey, 1991) and for IgG as described in section 3.3.3.1. The Con A assay was as described in 4.5.3.1, although the fractions were sampled (10 µL) without dilution.

b. Stepped Elution with Additional Washes

The previous gradient elution was modified: only the pH buffers which had eluted protein were used; twice the volume of these was applied to ensure all possible IgG was

eluted with each buffer, and additional washes were applied after the sample application to ensure that the protein eluted with pH 7.8 was not simply flow-through of the sample.

Fractions were collected into 0.1 mL of neutralising/preserving buffer. 0.5 mL of CSF PB (containing 33 mg/L IgG) was mixed with 0.5 mL adsorption buffer and applied to the column with collection of the breakthrough (Fraction1). Three washes of adsorption buffer were applied with collection of breakthrough after each (Fractions 2 to 4). Then 0.4 mL of pH 7.8 was applied (Fraction 5), 0.4 mL of wash (Fraction 6), two applications of 0.4 mL of pH 2.8 buffer (Fractions 7 and 8), two applications of pH 2.1 buffer (Fractions 9 and 10) and two applications of pH 1.6 buffer (Fractions 11 and 12). Total protein, IgG and Con A binding were again measured on the fractions as described in (a) above.

c. Stepped Elution with Small Volumes

0.2 mL of CSF was mixed with 0.2 mL adsorption buffer and applied to the column and the breakthrough collected (Fraction 1). The column was washed with 1.6 mL of adsorption buffer (Fraction 2) and a further 0.4 mL (Fraction 3). Then 0.4 mL of pH 7.8 buffer containing 2 M glycine was added (Fraction 4) followed by 0.4 mL of adsorption buffer (Fraction 5), 0.4 mL of pH 7.8 buffer (no glycine) (Fraction 6), and two additions of 0.4 mL of adsorption buffer (Fractions 7 and 8). Then 10 aliquots of 0.1 mL of eluate (pH 2.8 buffer) were applied with collection after each (Fractions 9 to 18). Finally 0.4 mL of pH 1.6 buffer was added (Fraction 19). To fractions 9 to 19 was added 25 µL of neutralising/preserving buffer.

Fractions 3 to 19 were assayed in duplicate for IgG and fractions 3 to 17 were assayed in triplicate for Con A binding, as described in (a) above.

4.5.3.3. Stability of IgG in Eluates

a. Without BSA

CSF BW (IgG = 172 mg/L) was fractionated with the Prosep column by applying 39 µL of the sample prediluted with 461 µL of adsorption buffer. (The volume applied was calculated from the [IgG] so that it contained 6.7 µg of IgG, which would produce an eluate containing 5 mg/L IgG, assuming 67% recovery). After draining, a further 0.8 mL of adsorption buffer was applied. After draining, 0.1 of elution buffer was applied followed by a further 0.8 mL of elution buffer with collection of the eluate into a glass bottle (z5) containing 0.2 mL of neutralising buffer. The column was cleaned with 0.4 mL of pH 1.6 buffer and re-equilibrated with 0.8 mL of adsorption buffer.

100 µL of the eluate was placed in a glass bottle at -70°C (Day 0). The remainder of the fraction was stored at 4°C and after 29, 40, 57 and 75 days, further 100 µL aliquots were placed at -70°C. These were thawed out and immediately diluted 1:5 with PBS and assayed for IgG as described in 3.3.3.1.

To test the stability of IgG in different solutions, 100 µL of 100 mg/L IgG stock solution was added to i) PBS, ii) pH 2.8 buffer and iii) pH 2.8 buffer containing neutralising/preserving buffer in the ration of 4+1. The mixtures were incubated for 1 h before assaying for IgG.

b. Stability of IgG Fractions in Different Bottles.

Calculated volumes of CSF P (containing 31 mg/L IgG), CSF B (366 mg/L IgG) and CSF L (66 mg/L) were diluted to 1.5 mL with adsorption buffer so that they contained 6.7 mg/L of IgG. (The volumes were 645 µL, 75 µL and 303 µL respectively). For each CSF 0.5 mL of diluted CSF was applied to the column and fractionated as described in a) above. This was repeated three times, pooling the eluates, for each CSF,

then adding a further 0.4 mL of elution buffer and 0.1 mL neutralising buffer to make up the volume to 3.5 mL.

The pooled eluates for each CSF were then aliquoted into containers as follows:

- 0.5 mL into glass, polystyrene or polypropylene bottles.
- 0.5 mL into similar bottles to which had been previously added 5 µL of 300 g/L BSA.
- 0.1 mL into two polypropylene tubes and placed immediately at -70°C.
- After 4 days further 0.1 mL aliquots were placed in polypropylene tubes and immediately frozen at -70°C.

The stored fractions were diluted 1 in 5 immediately before the IgG assay, however one set of the polypropylene-stored eluates was diluted 1:5 at 1 h before the assay, to see if IgG bound to the tube at this stage. The 'parent' CSFs P, B and L were diluted 1 in 31, 1 in 366 and 1 in 66 respectively. For the IgG assay the sample volume was 5 µL, with sampling in triplicate, otherwise the assay was performed as described in 3.3.3.1.

To measure IgG bound to the inside of the storage tubes, a rudimentary calibration curve was prepared by incubating IgG standard solutions (0, 0.2, 0.6, 1.0, 2.0 and 3.0 mg/L IgG) overnight in coated tubes, and washing. The tubes containing eluate, and those used to make dilutions, were aspirated and washed. Anti-IgG-HRP was diluted 1/2000 with PBS-Tween and 400 µL was added to all washed tubes and incubated for 45 min. After washing, 400 µL of prepared OPD solution was added to the tubes, as well as a blank tube, and incubated in the dark at room temperature for 30 min. After addition of 100 µL of 3 M HCl to each tube and mixing, the absorbance of the solutions was measured against the blank tube at 492 nm.

4.5.4. Results

4.5.4.1. Results for Preparation of the Column

a. Initial Con A Assay of Protein A Fractions

Con A binding of the CSF fractions is shown in Figure 4.23. CSF P appears to have proportionally less interferent binding than CSF B, as shown by the blocked-only tubes. In the breakthrough fractions P-1 and B-1, the proportion of interferent to IgG binding is as high or higher than in the untreated CSFs. However, in the HCl eluted fractions, the binding of interferent is much reduced. These results are encouraging and suggest Protein A may be used to eliminate the interferent.

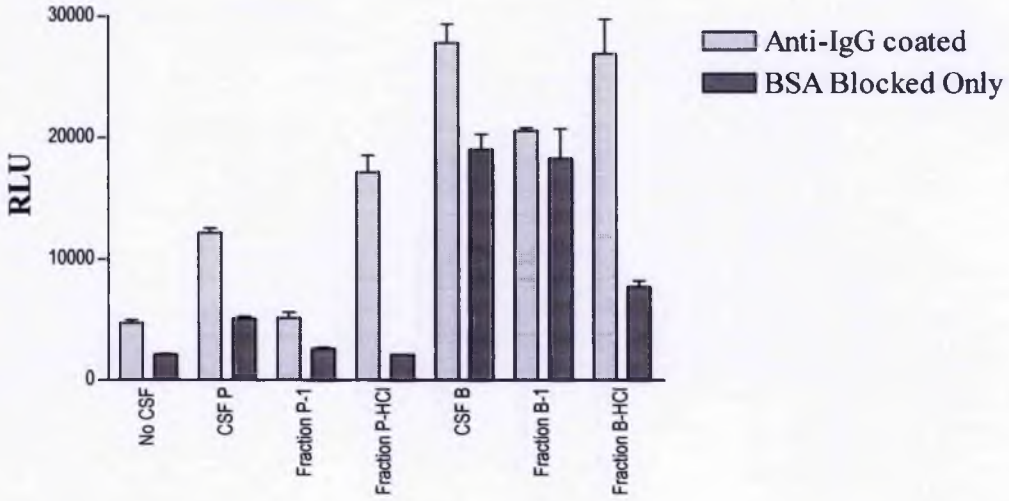


Figure 4.23. Con A assay of two CSFs and their protein A fractions. Error bars represent SEM of triplicate sampling in the assay. For experimental details see 4.5.3.1(a).

b. Adsorption Buffer

The result of the IgG (IT) assays of the eluate with different adsorption buffers is shown in Figure 4.24. The addition of glycine appears to dramatically improve the efficiency of the column.

c. Elution Buffer

Figure 4.25 shows the results of the IgG (IT) assays for different eluates. The pH 3.9 and 4.9 buffers are not of low enough pH to elute IgG at all. The chaotropic agents,

potassium iodide and sodium thiocyanate, also do not elute IgG. The pH 1.5, 2.15 and 2.8 buffers are equally effective, but the inclusion of glycine reduces elution.

d. Column Capacity

The column was loaded with 14 x 100 µL aliquots of CSF, but IgG was undetectable in each breakthrough volume collected after each 100 µL load. This indicated that the 106 µg in total of IgG loaded onto the 0.2 mL column did not exceed the column

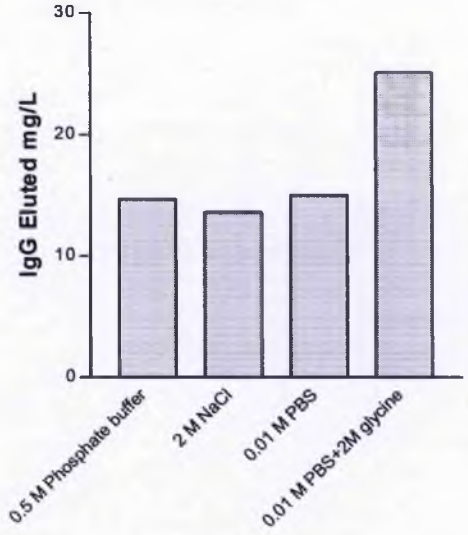


Figure 4.24. The effect of different adsorption buffers on IgG elution. For experimental details see 4.5.3.1(b).

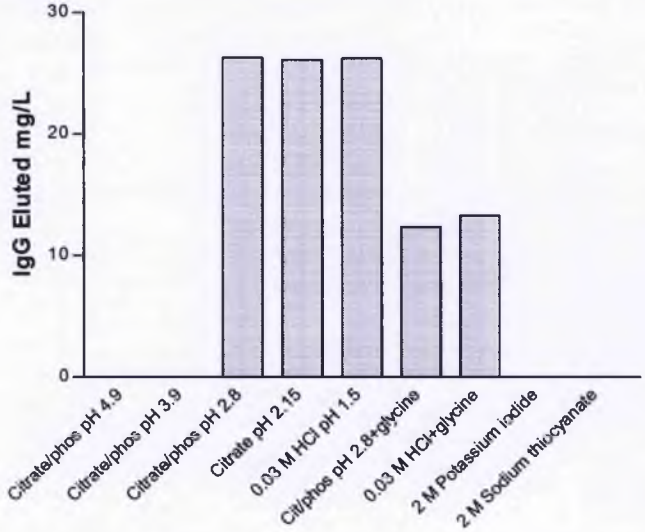


Figure 4.25. Elution of IgG with Different Elution Buffers. For experimental details see 4.5.3.1(c).

capacity. This is far above the level (6.7 μg) that will in practice be loaded onto the column, and far below the manufacturers stated column capacity of 11 mg of human IgG per 1 mL column (Godfrey, 1996).

4.5.4.2. Results for Stepped Elution Studies

a. Stepped Elution with Buffers of Decreasing pH

Figure 4.26 confirms the findings of 4.5.4.1(c) that IgG is not eluted until pH 2.8 is used. However, even though the recovery of IgG in the pH 2.8 fraction is 97%, there appears to be some IgG remaining which is eluted only with the HCl at pH 1.6. It is possible that this IgG would have been eluted with more pH 2.8 buffer, and this will be checked in the next experiment. This fraction also has a high amount of Con A binding relative to the IgG concentration compared to the pH 2.8 fraction, suggesting the IgG in this fraction is glycosylated differently from that in the pH 2.8 fraction.

Some non-IgG protein material, which binds to a small extent to Con A, is eluted with pH 7.8. In the next experiment the volume of wash buffer will be increased to try to remove this.

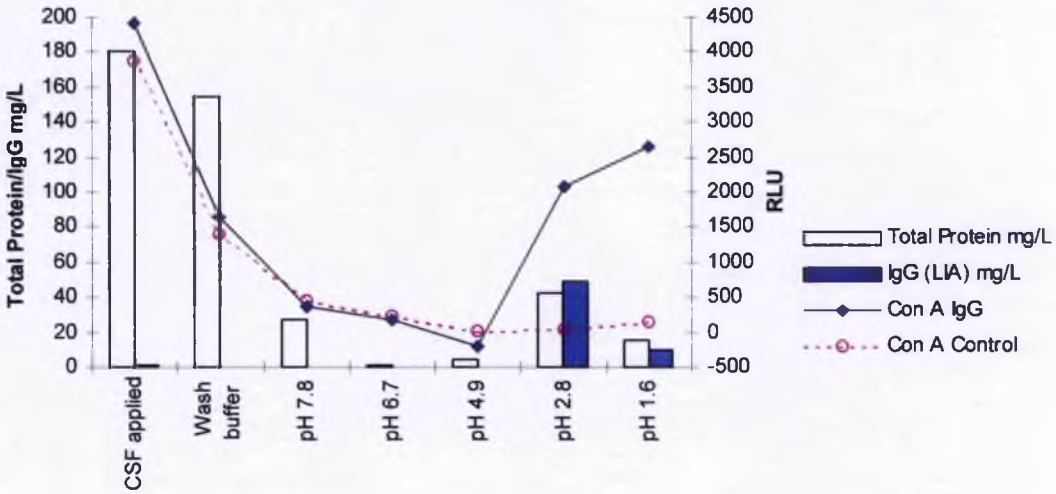


Figure 4.26. Stepped Elution 1. Measurement of fractions of CSF eluted from a protein A column using, stepwise, buffers of decreasing pH. For experimental details see 4.5.3.2(a).

b. Stepped Elution with Additional Washes

Figure 4.27 shows that the majority of IgG is eluted with the two volumes of pH 2.8 buffer. Therefore in the first stepped elution experiment the IgG appearing in the 1.6 pH buffer (Figure 4.24) was due to insufficient volume of 2.8 buffer to elute all bound IgG, and was not a function of the lower pH. Interestingly, the Con A binding relative to IgG in the second IgG fraction was higher than in the first fraction in both experiments.

Figure 4.27 also shows that protein is still being removed from the column even in the fifth wash, although in this wash the interferent binding is minimal. The pH 7.8 buffer, however does appear to elute some Con A binding material, and may be due to the different pH, different molarity or lack of glycine, compared to the adsorption buffer.

The IgG recovered in fractions 7 and 8 combined is 91% of the IgG applied to the column.

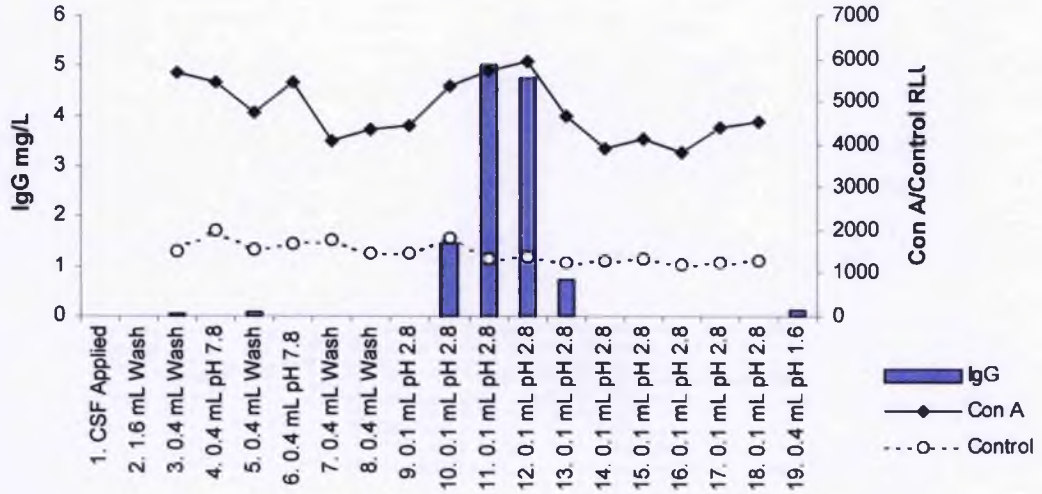


Figure 4.28. Stepped Elution 3. Elution of IgG from a protein A column with small volumes (0.1 mL) of pH 2.8 buffer, with additional washes before elution. For experimental details see 4.5.3.2(c).

c. Stepped Elution with Small Volumes

Figure 4.28 shows that the majority of IgG is eluted with 0.4 mL of pH 2.8 buffer, after addition of the first 0.1 mL when no IgG is eluted. There are small amounts in the wash fractions, and notably fraction 5 due to addition of pH 7.8 buffer with glycine. There is no additional IgG eluted in fractions 6 or 7 from the pH 7.8 buffer with no glycine, therefore it appears to be the pH change which elutes IgG with this buffer, rather than a change in molarity.

Fraction 19 contains some IgG eluted with the pH 1.6 buffer, but is only 0.4% of the IgG applied to the column, so it should not affect the results of the lectin assays. The recovery of IgG in fractions 10, 11, 12 and 13 combined was 53%.

The Con A binding of the control tubes indicates that there is very little interferent in this particular CSF. The Con A binding with the antibody coated tubes shows high levels with the washes (from non-IgG material) and a peak with the IgG peak.

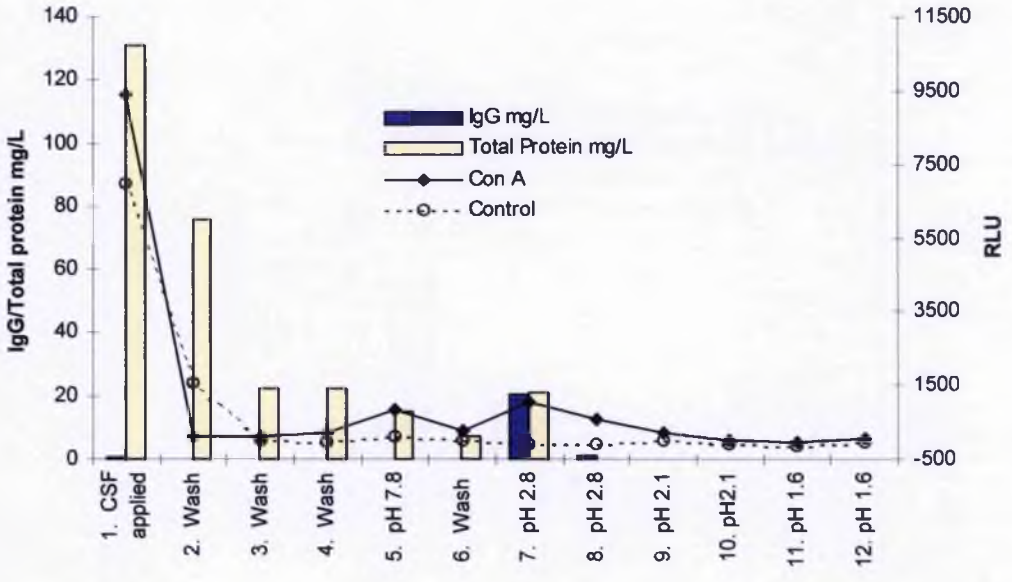


Figure 4.27. Stepped Elution 2. Measurement of fractions of CSF eluted stepwise from a protein A column, with incorporation of additional washes. For experimental details see 4.5.3.2(b).

However there is no indication of a gradient of glycosylation of IgG as was suspected.

4.5.4.3. Results for Stability of IgG in Eluates

a. Without BSA

Measurement of the fresh eluate gave an IgG level of 4.23 mg/L. This was considerably higher than the levels in the aliquots frozen after storage at 4°C for between 0 and 75 days, as shown in Figure 4.29. Freezing appears to have an adverse effect on IgG, although there is also a loss over time at 4°C. This shows that addition of neutralising buffer to the eluates preserves IgG in the short term.

After storage at 4°C for 1 h, the 10 mg/L IgG diluted in PBS was 9.97 mg/L, in the pH 2.8 buffer it was 6.9 mg/L and in the pH 2.8 buffer with neutralising buffer, it was 10.4 mg/L.

b. In Different Containers

Figure 4.30 shows the IgG measured in the different bottles after storage of eluates for 5 days. It is apparent that addition of BSA greatly improves recovery of IgG. Freezing drastically reduces IgG levels. ANOVA tests (Bonferroni's Multiple Comparison

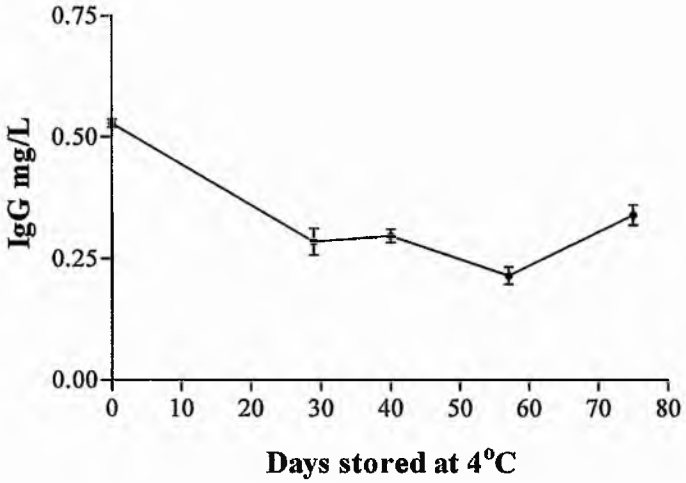


Figure 4.29. Stability of IgG in eluate, without addition of BSA, stored at 4°C. Error bars represent SEM of triplicate samples. For experimental details see 4.5.3.3(a).

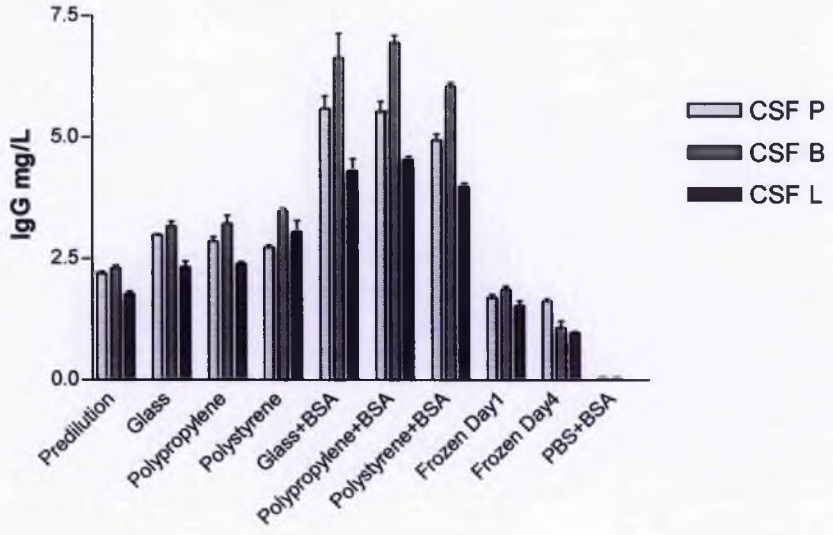


Figure 4.30. Measurement of protein A eluates from three CSFs, stored for 5 days at 4°C (or frozen) in different containers with and without addition of BSA. Error bars represent SEM of triplicate sampling in the IgG assay. For experimental details see 4.5.3.3(b).

Tests) showed no significant difference between the means for samples stored in glass, polypropylene and polystyrene without BSA (P=0.6771), or for them stored with BSA (P=0.7560). Paired t test comparisons of 'glass' with 'glass with BSA', and 'polypropylene' with 'polypropylene with BSA' showed the means to be significantly different (P=0.0248 and 0.0256 respectively) although comparison of 'polystyrene' with 'polystyrene with BSA' showed no difference (P=0.0608). Paired t tests comparing the frozen samples (day 1) with the polypropylene samples showed a difference (P=0.0160), as did comparison of the prediluted samples with samples ('glass') diluted just before assaying (P=0.0153).

Figure 4.31 shows the results of the OPD assay of IgG bound to the containers. The binding to polypropylene without BSA addition was much higher than to the other materials. Addition of BSA almost completely reduced IgG binding to polypropylene and polystyrene, but with glass it remained the same. There was a small amount of IgG binding to the glass bottles used for the dilutions.

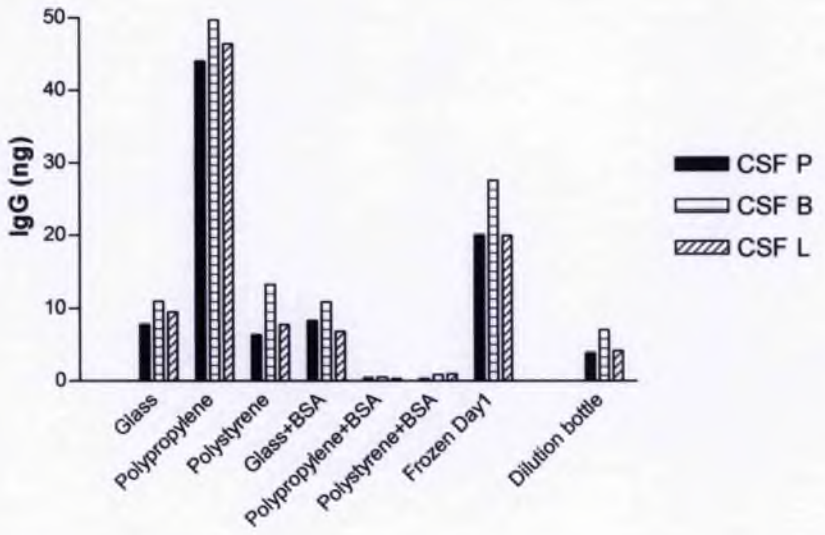


Figure 4.31. Eluate IgG bound to different containers with and without addition of BSA after storage for 5 days. IgG was measured using anti-human IgG-HRP conjugate with OPD/peroxide substrate. For experimental details see 4.5.3.3(b).

However the amount of IgG bound to the containers, from about 10 ng up to 50 ng (Figure 4.31), is small compared to the amount of IgG in solution (Figure 4.30). The IgG in solution, for 0.5 mL volumes of eluate that were placed in the containers ranged from about 1.25 μg for the eluates without BSA to a maximum of 3.5 μg with BSA. - about 100 times more than the IgG measured on the containers. For the highest binding tube (polypropylene without BSA), the IgG bound to the tube is about 2.5% of the IgG measured in solution.

Recoveries of IgG applied to the column were 80%, 80% and 67% for eluates of CSFs P, B and L respectively in glass bottles with BSA, compared to 42%, 37% and 35% for the same eluates in glass without BSA.

4.5.5. Discussion for Protein A Separation of IgG

A method for affinity purification of IgG was developed for the lectin assays, using a 0.2 mL column of protein A attached to glass beads. For the adsorption buffer, addition of 2 M glycine increased recovery of IgG. For elution, citrate-phosphate buffer

of pH 2.8 was found to be as effective as lower pH eluants. Chaotropic agents did not elute IgG (possibly due to interference by glycine).

The amount of IgG applied to the column was far below the column capacity, giving optimum chance of recovery of all IgG subclasses, including IgG3.

Stepped elution studies showed that non-IgG material can be washed through the column prior to elution of IgG. The majority of IgG can be eluted from the 0.2 mL column with 0.4 mL of eluant, after a 0.1 mL 'prime' of eluant with the breakthrough not being collected. Recoveries of IgG ranged from 50% to 90%. The eluates were largely free of lectin-binding interferents, as determined by assays using blocked-only control tubes. Con A binding of the elution fractions suggested that IgG eluted as a gradient of glycoforms, but this was not confirmed.

Collection of the eluates onto neutralising 0.5 M phosphate buffer reduces loss of IgG owing to the low pH of the eluant. Some loss of IgG was found to be due to binding to the storage container, but most would appear to be from denaturation or aggregation of the IgG. Addition of BSA was found to reduce the loss, possibly fulfilling a protective role towards the IgG in the same way as proteins would in the body. Freezing of the eluate has an adverse effect on IgG. Therefore eluates will be stored in glass bottles with added BSA at 4°C for up to 2 weeks before lectin assays are performed. For the lectin assays, the desired IgG content of each eluate is 5 μg per 1.0 mL of eluate. Recovery of IgG from the column in earlier studies had averaged 75%, therefore the IgG to load on the column is 6.67 μg, and the volume of a CSF sample to apply will be:

$$\frac{(6.67 \times 1000)}{[\text{IgG}] \text{ of sample}} \quad \mu\text{L}.$$

Measurement of IgG in the eluates will then enable adjustment of each sample volume for the lectin assays, so that a consistent amount of IgG from each sample is available for binding by the capture anti-IgG.

4.6. Lectin Assays of MS and Control CSF samples

4.6.1. Introduction

The foregoing work has enabled the development of a working method for probing sugars on CSF IgG using lectins, as will be described. The method was used to analyse two groups of samples: CSF from patients with confirmed MS, and control CSF from neurological patients without demyelinating disease. Four different lectins were used, selected by showing binding to CSF IgG in the development work, as well as from information from the literature.

4.6.2. Reagents

See previous Reagent sections for full descriptions.

Affinity Chromatography:

- Prosep protein A column, 0.2 mL.
- Phosphate buffered saline 0.01 M ('PBS'). Sigma PBS tablets.
- Adsorption/wash buffer. 0.01 M PBS containing 2 M glycine, pH 7.0.
- Elution buffer. 0.05 M solutions of citric acid and Na_2HPO_4 mixed to produce pH 2.8.
- Neutralising/preserving buffer. 0.5 M phosphate buffer containing NaN_3 (5 g/L) and BSA (7.5 g/L).
- HCl 0.03 M, pH 1.6.

IgG/Lectin Immunoassays:

- Biotinylated lectins. From Vector Labs. Reconstituted as recommended to give concentrations of 5 mg/L (Con A and RCA_{120}), 2 mg/mL (SNA) and 1 mg/mL (DSL). Aliquots of 50 µL were stored at -70°C.
- Lectin assay buffer. Made up freshly before use. TBS (Tris buffered saline tablets, Sigma Co.) containing 1 M $MgCl_2$ and 1 M $CaCl_2$.

- IgG assay buffer. PBS containing Tween (0.5 g/L)
- Coating buffer. Carbonate buffer pH 9.2 (Sigma Carbonate buffer tablets) containing anti-IgG (Sigma) diluted 1:400 (for the IgG assays) or 1:100 (for the lectin assays).
- Wash solution. NaCl (9 g/L) containing Tween (0.5 mL/L).
- Blocker. 10 g/L BSA. 32 mL PBS containing 0.96 mL of 300 g/L BSA.
- Periodate 0.01 M. 63 mg of $NaIO_4$ in 30 mL citrate buffer, 0.05 M pH 4.0. Prepared freshly before use.

4.6.3. Samples

MS positive samples were selected on the basis of having a) intrathecally formed oligoclonal IgG bands on IEF, (b) elevated CSF IgG and percentage of CSF total protein, (c) an MRI scan implicating demyelination, (d) clinical diagnosis of 'MS' or 'probable MS'.

Control samples were selected as having (a) oligoclonal bands negative, (b) normal results for CSF total protein, IgG, glucose and white cell count (<5), (c) no evidence of demyelination on MRI scan, (d) MS excluded from clinical diagnosis.

Using these criteria, 13 samples were selected as MS positives and 14 as controls and stored at -70°C for up to 6 months.

4.6.4. Method

4.6.4.1. Affinity Chromatography

1. For each sample, the amount of CSF (in μL) to load onto the column was calculated from the formula (6.67 x 1000)/[IgG]. The calculated volume of CSF was diluted with adsorption/wash buffer to a volume of 0.5 mL and applied to the column.
2. After draining, 0.8 mL of adsorption wash buffer was applied to the column.
3. After draining, 0.1 mL of elution buffer was applied.

4. After draining, 0.8 mL of elution buffer was applied and the eluate collected onto 0.2 mL of neutralising/preserving buffer in a 5 mL glass bottle.
5. 0.4 mL of pH 1.6 buffer was applied to the column and allowed to drain.
6. 0.8 mL of adsorption/wash buffer was applied to the column and allowed to drain.
7. Steps 1 to 6 were repeated for each sample.

4.6.4.2. IgG Immunoassay

The IgG method was as described in section 3.3.3.1. CSF samples were diluted 1:40 and the eluates 1:5 with PBS-T immediately prior to the assay.

4.6.4.3. Lectin Immunoassays

Polystyrene 'star' tubes were coated overnight with 0.2 mL of anti-IgG diluted 1:100 with coating buffer, then blocked with 0.3 mL of 10 g/L BSA for 1 h. The antibody coating was deglycosylated with 0.3 mL of 0.01 M sodium periodate in citrate buffer (0.05 M, pH 4.0), incubating for 20 h at 4°C.

For each eluate, the sample volume (ranging from 5 to 15 μL) was calculated from the result of the IgG immunoassay, so that 50 ng of IgG was added to each assay tube. 0.2 mL of PBS-T was added to the prepared/periodate-treated tubes, followed by the prescribed volume of eluate, in triplicate tubes. After incubation for 1.5 h, tubes were washed and to each was added 0.2 mL of biotinylated lectin, diluted to 500 μg/L with lectin assay buffer. After incubation for 1.5 h and washing, 0.2 mL of isoluminol-streptavidin, 10 μL diluted in 100 mL of lectin assay buffer, was added, followed by further incubation for 1 h, washing and measurement of relative luminescence units.

4.6.5. Results for Lectin Assays of MS and Control CSF Samples

Figures 4.32 to 4.35 show for each lectin the binding to IgG in the MS samples compared to the controls. It can be seen that for both Con A and RCA, and to a lesser extent for SNA, there is a wide scatter of results for the MS samples, with a tight bunching for the controls. Comparison of the means of the two groups for each lectin gave the results shown in Table 4.3, using an unpaired t test, two-tailed, with Welch's correction (for unequal variances). Although only Con A showed significant difference between the means at the 95% level), the P value for RCA is very low. With DSL and SNA there is no difference between the means. Variances of the two groups were significantly different for Con A and RCA ($P<0.0001$) and SNA ($P=0.0012$), but not for DSL ($P=0.4011$).

Table 4.3. Unpaired t test of MS samples v controls.

Lectin-binding	MS: RLU Mean ± SEM (n = 13)	Controls: RLU Mean ± SEM (n = 14)	P value
Con A	4275 ± 906	1716 ± 244	0.0172*
RCA	1220 ± 375	533 ± 87	0.0981
DSL	514 ± 95	459 ± 98	0.6922
SNA	6445 ± 896	5701 ± 345	0.4506

* Means significantly different ($P<0.05$) between MS and controls.

The bindings of the four lectins for all samples were analysed for correlation with each other, and with CSF [IgG], CSF [total protein], and CSF (IgG/total protein)%, as shown in Table 4.4. Con A binding was found to correlate with %IgG ($P=0.0222$) and, more strongly, RCA binding with SNA binding ($P<0.0001$).

To compare the lectin bindings of each sample, results for Con A, RCA and SNA were normalised by converting the RLUs to percentages: the lowest RLU taken as 0% and the highest 100% for each lectin, and the intermediate results calculated accordingly. The normalised results are plotted in Figure 4.36, comparing Con A with RCA, and Figure 4.37, comparing RCA with SNA. The MS positive samples are indicated by an asterisk. Looking at Figure 4.36, the three highest Con A results are for MS samples and the three highest RCA results are for different MS samples, suggesting a negative correlation, but otherwise there is no obvious trend. With Figure 4.37, the correlation between RCA and SNA ($P<0.001$) can be distinguished, but no other patterns can be seen.

Table 4.4. Correlations of lectin binding

Groups Compared	Correlation Coefficient (r)	P-value
Con A v RCA	0.0650	0.7474
Con A v DSL	0.1755	0.3811
Con A v SNA	-0.0203	0.9217
Con A v [IgG]	0.2263	0.2564
Con A v [total protein]	0.0010	0.9959
Con A v %IgG	0.3900	0.0443*
RCA v DSL	-0.0467	0.8171
RCA v SNA	0.7924	<0.0001*
RCA v [IgG]	0.0882	0.6617
RCA v [total protein]	0.0674	0.7385
RCA v %IgG	0.2034	0.3088
DSL v SNA	-0.0532	0.7964
DSL v [IgG]	0.1048	0.6029
DSL v [total protein]	-0.0906	0.6533
DSL v % IgG	0.2888	0.1440
SNA v [IgG]	-0.1023	0.6194
SNA v [total protein]	-0.1506	0.4628
SNA v %IgG	0.1281	0.5329

*Pairing significantly effective.

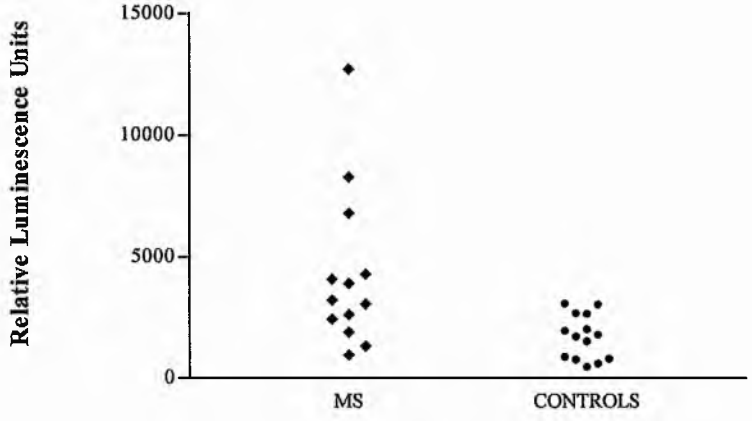


Figure 4.32. Con A binding of MS samples compared to controls.

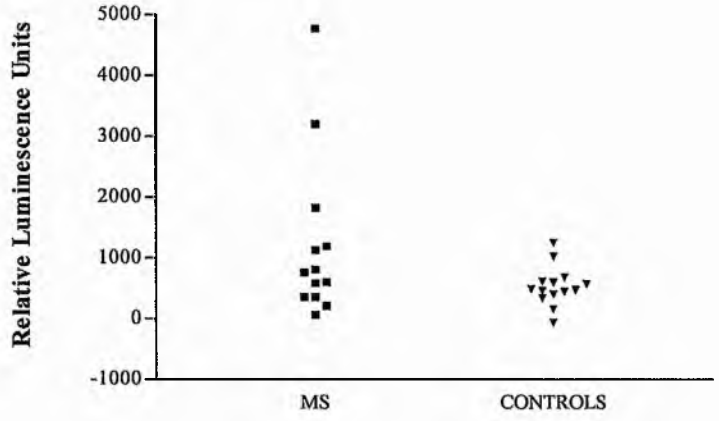


Figure 4.33. RCA binding of MS samples compared to controls.

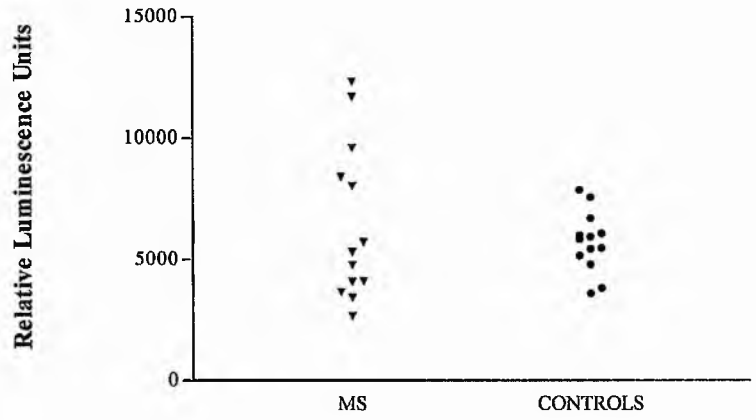


Figure 4.34. SNA binding of MS samples compared to controls.

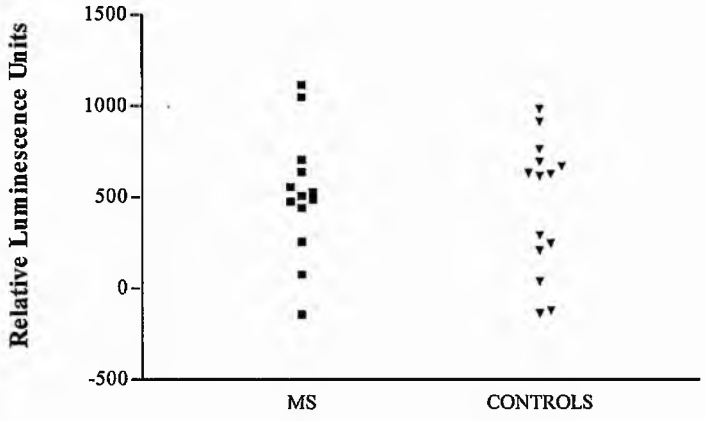


Figure 4.35. DSL binding of MS samples compared to controls.

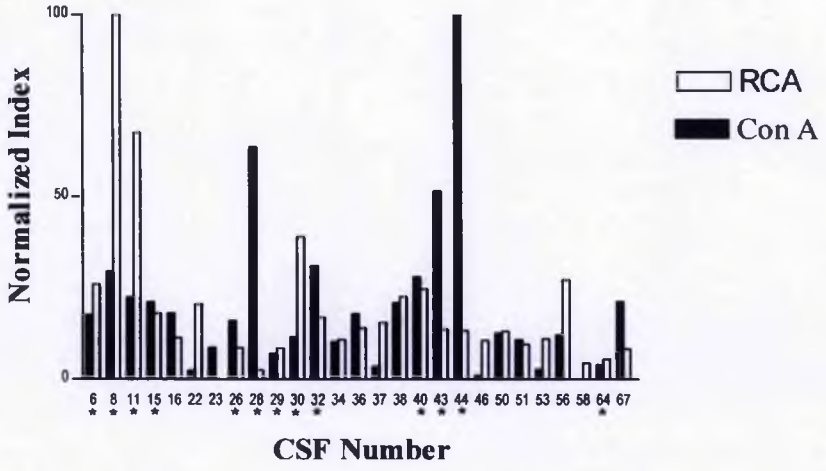


Figure 4.36. Comparison of results of RCA and Con A binding for each sample. * Indicates MS samples.

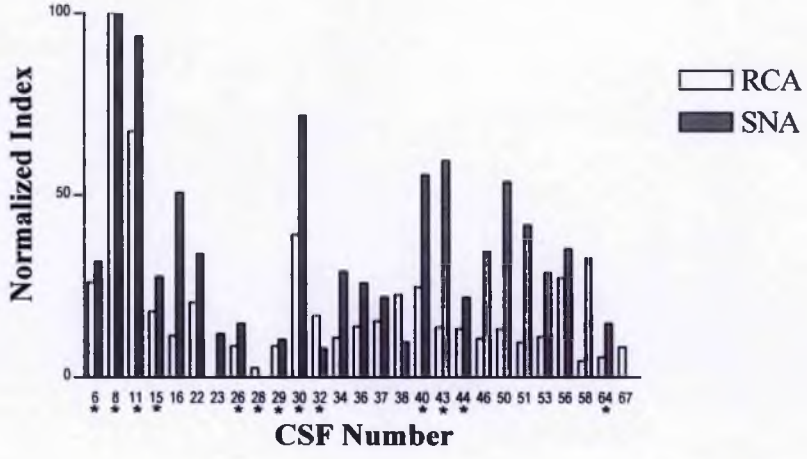


Figure 4.37. Comparison of results of RCA and SNA binding for each sample. * Indicates MS samples.

4.6.6. Discussion for Lectin Assays of MS and Control CSF Samples

Of the four lectins, Con A showed the most significant differences between the MS samples and controls. The means are significantly different, with a wide spread of higher binding for the MS samples. There is also a positive correlation with %IgG, not seen with RCA, which is probably to be expected if Con A does distinguish between MS and non-MS, because %IgG is known to correlate with MS and is used clinically as a marker for the disease.

RCA also shows a wider and higher spread with MS samples, even though the means of the two groups are not quite significantly different for the numbers of samples used. The three very high RCA results are MS positives and also have high Con A binding, but overall there is no correlation between the two lectins.

SNA and DSL do not distinguish between MS samples and controls. However, there is a strong correlation between SNA and RCA binding, which suggests that where sialic acid is lacking, galactose is also deficient. The increased SNA binding in a large proportion of the MS samples (Figure 4.34) does not support the theory that oligoclonal IgG is deficient in sialic acid.

Con A generally shows higher RLUs than the other lectins and shows a degree of binding in all samples - i.e. all samples contain mannose. RCA, however, and DSL and SNA even more so, have zero or virtually zero binding with some samples. It is feasible that some samples contain IgG with little sialic acid or with little galactose, but DSL is present in all IgG glycans. This suggests that steric hindrance might affect DSL binding, as discussed in section 4.7.

There are several possible reasons for the increased Con A binding in the MS samples: there could be an overall increase in glycans attached to IgG in MS samples, or there could be an increase in the branching of the glycans in MS. Either of these would

be consistent with the increases, albeit not significant, in SNA and RCA binding seen with MS samples.

4.7. Development of Lectin Binding Assays: Overall Discussion

Reliable lectin immunoassays were eventually developed to probe the oligosaccharides of CSF IgG. Because of the problem of glycosylated substances apart from IgG binding to the capture antibody, there needed to be a preliminary separation of IgG from CSF and this was finally achieved by using protein A affinity chromatography.

The main findings of the lectin assays were:

- Con A showed most significant difference between MS samples and controls.
- Con A showed a positive correlation with %IgG, which is used clinically as a marker for MS.
- The increased Con A binding in MS samples - i.e. increased mannose - suggests either overall increase, or increased branching, of IgG glycans in MS.
- The strong correlation between SNA and RCA binding is consistent with the understanding that where galactose is deficient, sialic acid must necessarily be lacking.
- The increased SNA binding in a large proportion of MS samples does not support the hypothesis that oligoclonal IgG is deficient in sialic acid.

There are several problems with using lectins to investigate the glycans of a protein. One is the range of binding specificities that each lectin has, although usually the relevant target can be deduced. Another is the possibility of steric hindrance to their binding by the protein, which can, however, be overcome by denaturation of the protein (Sumar et al, 1990). The relatively low binding generally of DSL, and possibly RCA, might be due to steric hindrance. The moieties which are bound by DSL - GlcNAc(β1-4)GlcNAc]$_{1-3}$ and Gal(β1-4)GlcNAc - are present in all glycan chains of IgG (found to

date) so binding to them would be thought to be as extensive as the binding to mannose by Con A. In the assays of four CSF samples with a panel of lectins, in which the CSFs were not affinity purified but were diluted to 10 mg/L of IgG, DSL certainly showed substantial binding in all CSFs (Section 4.3.4.7; Figure 4.12), comparable to the levels with SNA. However, there is more binding shown by DSL, compared to Con A, RCA and SNA, to the capture antibody (see the 'No CSF' chart, Figure 4.12). This binding, plus the interferent binding may explain the substantial binding seen with the capture antibody coated tubes for the CSFs in Figure 4.12.

Another possibility that should be considered is that the assay did not work properly although there was no reason to suspect this at the time. Although there are moves to produce an agalatosyl IgG control for assays for RA, finding controls for Con A, SNA and DSL binding is a problem. Running a serum sample would not help, unless it were known, from a previous reference assay, what level of lectin binding was expected. But, for comparison of one group of samples with another, in a single assay for each lectin, in the case of this project, where actual concentrations are not needed, a control does not seem essential. In a way, the non-coated tubes are controls, as are the tubes assayed with no CSF, and the tubes assayed with no lectin.

Overall, the lectin assays have shown that there is a difference in IgG glycosylation in MS CSF compared to controls. This may be due to variant glycosylation of oligoclonal IgG compared to polyclonal. There will also be polyclonal IgG in the MS samples, which will lessen the differences in glycosylation seen between the two groups. Thus it would seem that the spread of bands found in IEF of oligoclonal IgG may be due to differences of glycosylation, but it is more likely that the differences are in the peptides as well as in glycosylation. This should be made clearer by the methods of glycosylation analysis in the next chapters - namely IEF studies of IgG, and the more specific HPLC quantitation of IgG glycans.

Chapter Five

Isoelectric Focusing of CSF and Detection of Glycosylation using Lectins

5.1. Introduction

The intention was to use IEF to separate IgG bands and use lectins to detect differences in glycosylation of the bands.

Two IEF methods were used. Firstly, a routine method for detection of oligoclonal IgG bands using a gel prepared in-house, with a 7 to 10 pH gradient. Secondly, a method using bought, pre-cast gel strips with a 3 to 10 pH gradient. Although oligoclonal IgG runs mainly in the alkaline pH region, the wider pH range would allow examination of the total IgG fraction.

Polyvinylidene fluoride (PVDF) blots of CSF samples run by both methods will be shown, with visualisation by different methods. Also with the pH 3 to 10 system, blots of focused proteins of known pI were run, in order to calibrate the system. Finally, two samples of CSF IgG were deglycosylated, to compare the resultant bands with those of the whole CSF.

5.2. Reagents and Equipment

Reagents are as previously described unless listed below.

1. Polyacrylamide gel strips, pre-cast on plastic support film, with an immobilised linear 3 to 10 pH gradient. Size 110 x 3 mm. Immobiline DryStrip, Pharmacia

Fine Chemicals AB, Uppsala, Sweden, product code 18-1016-61. The strips are delivered in a dehydrated form and stored at -20°C. After rehydration, the gel thickness of the strips is 0.5 mm.

2. Reswelling cassette. Pharmacia, 18-1013-74.
3. Electrophoresis unit. Multiphor II, Pharmacia 18-1018-06.
4. Rehydration solution:
    - Deionized water 10 mL
    - Pharmalyte pH 3-10 Immobiline solution (Pharmacia 17-0456-01) 52 µL
    - Urea (Merck 10290) 5.4 g
    - Triton x-100 (Merck 30632) 52 µL of 0.5% (v/v)
    - 2-Mercaptoethanol (Merck 44143). 40 µL
    - Orange G (G. T. Gurr Ltd., London) 1 mg
5. Repel-Silane. Pharmacia 80-1129-42.
6. Wick solutions - 0.5 M H_2SO_4 for anode wick.
   - 1 M NaOH for cathode wick.
7. PVDF microporous membrane. Immobilon-P from Millipore Corporation, Watford, WD1 8YW, U.K. Product No. IPVH 000 10. 26.5 cm x 3.75 m roll; 0.45 µm pore size.
8. Slide-A-Lyzer dialysis cartridge. 3500 molecular weight cut-off; 0.1 to 0.5 mL capacity. Product No. 66335. Pierce Chemical Company: from Pierce and Warriner (U.K.) Ltd, Chester CH1 4EF.
9. Extavidin-horseradish peroxidase (Avidin-HRP). From Sigma Chemical Co., Poole, Dorset, BH12 4QH. Used at 1:2000 dilution, i.e. 20 µL + 40 mL PBS-Tween.

10. Hydrogen peroxide 6% (w/v) aqueous solution. Thornton and Ross, Huddersfield, HD7 5QH, U.K.
11. 3, 3'-Diaminobenzidine (DAB). D-5637 Sigma Co. **Caution: possibly carcinogenic.** For use, 10 mg of DAB was dissolved in 20 mL of TBS with addition of 50 µL of 6% (w/v) hydrogen peroxide.
12. DAB Tablets. 3, 3-diaminobezidine tetrahydrochloride, 10 mg. Kem-En-Tec A/S, Lersø Parkallé 42, DK-2100 København Ø, Denmark. Just before use, two tablets, after equilibration to room temperature for 15 min, were dissolved in 20 mL of water (giving a solution of pH 7.0 containing 1 mg/mL of DAB in 20 mM phosphate buffer) with addition of 100 µL of 6% (w/v) hydrogen peroxide.
13. pI calibration kit pH 3 - 10. Pharmacia. One vial was reconstituted with 0.5 mL of water, giving concentrations of each marker of between 40 to 100 ng per µL.
14. Peptide-N-glycosidase F. Oxford GlycoSciences (UK) Ltd. From *Flavobacterium meningosepticum.* Supplied as frozen 20 µL aliquots containing 4 Units.
15. Enzyme incubation buffer. Oxford GlycoSciences. Supplied five times concentrated, which when diluted with four volumes of water gives 20 mM sodium phosphate pH 7.5 containing 50 mM EDTA and 0.02% (w/v) sodium azide.
16. Igepal CA-630 (non-ionic detergent; analogue of Nonidet P40). Sigma Co. A 10% (v/v) solution was prepared by adding 0.1 mL of Igepal to 0.9 mL of working incubation buffer.
17. Sodium dodecyl sulphate (SDS). Electran grade, Merck Ltd.

18. Wash saline. NaCl (9 g/L) containing Tween (0.5 mL/L).

19. Trypsin inhibitor (soybean). Product no. T-9003, Sigma. pI =4.55

20. β-Lactoglobulin (bovine milk). L-2506, Sigma. pI = 5.20.

21. Carbonic anhydrase (bovine erythrocyte). C-2273, Sigma. pI = 5.85.

22. Myoglobin (horse heart). M-1882, Sigma. pI = 6.85, 7.35.

23. Lentil lectin (lens culinaris). L-9267, Sigma. pI = 8.15, 8.45, 8.65.

24. Trypsinogen (bovine pancreas). T-9011, Sigma. pI = 9.30.

25. Tetrachloroauric acid trihydrate. Sigma G-4022. A 10 g/L solution was prepared by dissolving 1 g in 100 mL water, stored in a dark container and kept cool.

26. Stannous chloride, anhydrous. Sigma S-2752. 10 g/L solution prepared freshly for use by dissolving 0.1 g stannous chloride in 0.4 mL of 1 M HCl and making up to 10 mL with water.

27. Citric acid, anhydrous. Sigma C-0759.

28. 5-Sulphosalicylic acid. GPR grade. Merck 30314.

29. Colloidal gold stain. Prepared as follows: 370 mL of water and 7.5 mL of 10 g/L tetrachloroauric acid were place in a dark container and 2.5 mL of 10 g/L stannous chloride solution was added dropwise with gentle stirring. Then, 110 mL of 10 g/L citric acid and 12.5 mL of Tween 20 were added, slowly with gentle mixing. After standing overnight in the dark, 25 g of anhydrous citric acid was added slowly with gentle mixing, followed by 2 g of sulphosalicylic acid with mixing. After 24 h the stain was ready to use, and could be used for up to 4 weeks if stored in a cool dark place.

5.3. Methods

5.3.1. Isoelectric Focusing with pH 3 - 10 Gradient

a. Rehydration of Gel Strips

Following Pharmacia's instructions, the gel strips, with protective film removed, were placed inside the reswelling cassette, which was then filled with rehydration solution. The cassette was tilted upwards at an angle of about 10°, in a refrigerator, and the strips allowed to rehydrate over 48 h.

b. Isoelectric Focusing

The rehydrated strips were aligned on the electrophoresis platform with the pointed end (pH 3) at the anode (red) electrode, and lightly blotted with dampened filter paper. CSF sample, from 1 to 4 µL, was usually applied in the middle of a strip keeping the application spot as small as possible. Paper wicks moistened with 0.05 M H_2SO_4 (anode) or 1.0 M NaOH (cathode) were placed across the ends of the strips, covering 3 mm of the gel. Pieces of blotting paper, cut to 10 x 4 cm, were placed at each end of the platform, just overlapping the each wick. The electrode wires were lowered onto the wicks at the points where they covered the gel. Dampened paper towels were placed on each side of the platform, the lid was closed and the water cooling turned on.

Several running conditions were tried. In each case 300 V was initially applied for 1 h, followed by: 1100 V for 6 h (6900 Volt hours in total); 950 V for 22 h (21200 Volt hours), or 1400 V for 15 h (21300 Volt hours).

After the run, the power was switched off, and the electrodes, blotters and wicks removed. The strips, *in situ*, were very lightly blotted with damp blotting paper.

c. Transfer to Membrane

PVDF membrane, cut to size, was immersed in methanol for 15 s, then water for 2 minutes, and then lightly blotted. It was laid over the strips, covered with damp blotting

paper, absorbent towels, then a glass plate, and then a 2 kg weight. After 1 h, the weight, blotter etc. were removed. The PVDF membrane was briefly immersed in water to remove the strips. The membrane could now be blocked and taken through a protein detection procedure. If the membrane was allowed to dry out, it needed to be rehydrated prior to blotting and detection by immersion in methanol (15 s) and water (2 minutes).

5.3.2. Isoelectric Focusing with pH 7 - 10 Gradient

All electrofocusing using pH 7 to 10 gradient gels described in this chapter was kindly performed by Dr Geoffrey Cowdrey in the Biochemistry Department at the Princess Royal Hospital. Details of the method are given in Cowdrey (1990). To summarise, the pH 7 to 10 acrylamide gels were prepared in house by mixing together, in a gradient former, two gels of differing densities and containing different concentrations of immobilines. Electrofocusing was carried out before sample application for 30 min at 1750 V, 50 mA and 10 W, and then after placing samples in formed wells, for 2 h at 5000 V, 10 mA and 10 W. Protein transfer onto PVDF membrane was as described in 5.3.1(c).

5.3.3. Detection Systems

5.3.3.1. Lectin

Each PVDF membrane containing separated proteins was immersed in PBS (20 mL) containing BSA (0.6 mL) for 1 h, or overnight at 4°C, in order to block remaining protein binding sites. Without washing, the membrane was transferred to 20 mL of lectin buffer containing 40 µL of biotinylated Con A or RCA and incubated for 1.5 h with gentle shaking. After washing three times in 40 mL wash saline for 5 min each with gentle shaking, the membrane was transferred into 20 mL of lectin assay buffer containing 10 µL of avidin-HRP and incubated for 1 h with gentle shaking. After washing as before, the membrane was immersed in 20 mL of DAB containing H_2O_2 (DAB either in tablet form or weighed out, as described in 5.2 Reagents and Equipment) and gently shaken for about 5 min until bands were seen to develop.

5.3.3.2. Anti-human IgG (Biotin-Avidin System)

The procedure was the same as 5.3.3.1 except 30 μL of biotinylated anti-human IgG was used in place of biotinylated lectin.

5.3.3.3. Anti-Human IgG (Double Antibody System)

This procedure, where used, was carried out by Dr Cowdrey. For details please see Cowdrey, 1990. Briefly, a membrane was blocked by immersion in a solution of dried skimmed milk, then incubated with anti-human IgG (raised in sheep). After washing, the membrane was incubated with donkey anti-sheep alkaline phosphatase conjugate, and after washing stained in buffer containing Nitroblue Tetrazolium with 5-bromo-4-chloroindoxyl phosphate.

5.3.3.4. Colloidal Gold

To visualise all transferred proteins, a PVDF membrane, without prior blocking treatment, was immersed in colloidal gold stain for 30 min with gentle shaking. It was then washed with water, and allowed to dry for at least 4 h at room temperature before viewing.

5.3.4. pI Calibration of pH 3 - 10 System

A vial of broad pI calibrators was prepared as described in 5.2(13) and run on pH 3 to 10 strips, according to the method of 5.3.1. Other proteins, namely carbonic anhydrase, myoglobin, lentil lectin, trypsinogen, trypsin inhibitor and β-lactoglobulin were dissolved in water (1 mg in 20 mL) and run with the broad pI calibrators in several assays. In all cases, 2 μL samples were applied to the centre of the gel strips, which were run at 300 V for 1 h, then 1400 V for 15 h. After blotting onto PVDF membrane, detection was with colloidal gold.

5.3.5. Deglycosylation of CSF IgG

5.3.5.1. First Sample

a. Affinity Chromatography

For the basic method, see 4.6.4. Here, 1.2 mL of CSF CE (Oligoclonal IgG positive; [IgG] = 94 mg/L) was loaded onto a 0.2 mL Prosep column along with 1.2 mL of adsorption buffer. After draining, the column was washed with 1.0 mL of adsorption buffer. After draining, 0.1 mL of elution buffer was applied to the column and drained. Then 0.5 mL of elution buffer was applied, and the eluate collected onto 0.1 mL of 0.5 M phosphate buffer, pH 7.4.

b. Dialysis

For a fuller description of the dialysis materials and method, see 6. . The eluate (0.6 mL) from *(a)* above was injected into a Slide-a-Lyzer dialysis cartridge and dialysed against three changes of 1.0 L of deionized water containing 0.7 mL of trifluoroacetic acid for 24 h each. The dialysate was removed and the cartridge washed with 0.2 mL of water, which was combined with the dialysate. The absorbance of the dialysate at 279 nm was measured against water and the IgG concentration calculated.

c. Denaturation

Denaturation of a protein is often recommended to afford the deglycosylating enzyme access to the glycan core (Hirani et al, 1987; Köttgen et al, 1993; Mann et al, 1994), although specific reference to IgG is not made. Therefore the deglycosylation was performed here with denatured and non-denatured (i.e. native) IgG.

0.2 mL of enzyme incubation buffer was added to the dialysate, which was then divided into 2 portions of 0.5 mL each.

To portion 1 (for denaturation) was added 10 µL of SDS (200 mg/mL), giving a solution of 0.4% (w/v) SDS. After mixing, 10 µL of mercaptoethanol was added and

mixed, to produce 2% (^{v}/v). The mixture was boiled for 2 min then cooled. 82 µL of Igepal was added and mixed. This mixture was divided into two equal aliquots: A and B.

Portion 2 (non-denatured) was divided into two equal aliquots (C and D).

d. Deglycosylation

One vial (4 Units) of PNGase F was added to each aliquot A and C. To B and D (controls) was added 20 µL of water. All aliquots were incubated for 24 h at 37°C.

e. Isoelectric Focusing

Isoelectric focusing was performed following the method described in 5.3.2 using gel with pH 7 to 10 gradient. 2 µL aliquots of the untreated CSF in triplicate, and aliquots of A, B C and D (C and D in duplicate) were applied to the gel. Detection was with the anti-human IgG double antibody system (5.3.3.3) or with biotinylated Con A (5.3.3.1).

5.3.5.2. Second Sample

Using a different sample, CSF NP (Oligoclonal IgG positive; [IgG] = 120 mg/L), the assay was repeated with some changes, as follows. 1.7 mL of the CSF was loaded onto the column with 1.7 mL of adsorption buffer. Washing of the dialysis cartridge was omitted, and the dialysate was mixed with enzyme incubation buffer to give a volume of 0.7 mL. Denaturation of the IgG was omitted. Double the enzyme as previously was used to ensure complete deglycosylation: the dialysate was divided into two equal aliquots, A and B; to A was added two vials (8 Units) of PNGase F and to B, 40 µL of water. Also an enzyme 'blank' was prepared by mixing 5 µL of buffer concentrate with 20 µL of water, and mixing 9 µL of this with 1 µL of PNGase F.

5.4. Results

5.4.1. Results for Isoelectric Focusing with pH 3 - 10 Gradient

It was found that the gel strips needed the 21000 Vh run time, as recommended by Pharmacia, and running at 1400 V for 15 h overnight, after 300 V for 1 h, was found to be most convenient. It was also found that better results were obtained if dampened tissues were placed in the tank to maintain humidity, rather than pouring in water. Condensation of water onto the gel was a problem, but could be reduced by placing blotting paper strips on top of each of the wicks.

Figure 5.1 shows the PVDF blot of pH 3 - 10 gel strips, stained with colloidal gold. Samples A, B and C are the eluate from the Prosep A column containing 1500 mg/L, 150 mg/L and 0 mg/L of BSA. No bands attributable to IgG can be seen, so the method does not appear to be sensitive enough to detect IgG in the eluate samples. The BSA gives a dense cluster of bands at around pH 6, clearly seen in strip D, which is BSA in buffer only. The broad range calibrators are run as strip E and, with added BSA, as strip F. The α-and β-lactoglobulin bands are clearly seen in strip G, and enable the β-lactoglobulin band in the calibrator to be positively identified. The bands for the other protein markers in the calibrator were identified by subsequent IEF runs of the broad pI calibrator alongside the pure proteins. The pI values of these markers were plotted against the distance from the cathode of their respective bands (Figure 5.2). The graph confirms that the pH 3 to 10 gradient is linear for the running conditions used.

Inability to visualise bands in the Prosep eluates was a problem with the pH 3 - 10 gel strips with lectin detection as well as with colloidal gold. Increasing the sample volume of eluate applied to the gel caused overheating due to focusing of the buffer salts in the sample, and also occurred without the incorporation of BSA. It would probably be

possible to run the eluates if salts were removed by dialysis, but the easiest solution would be to only run the whole CSF samples.

Figure 5.3 shows the PVDF blots of various whole CSF samples run on pH 3 - 10 gel strips. Blot 1 shows the bands for a CSF sample (W) negative for oligoclonal IgG by routine IEF, and for an oligoclonal positive sample (S), detected using Con A. The bands in sample S are possibly more extensive than in W. There is some faint staining in the alkaline region in sample W, which must be due to glycoproteins other than IgG. The large number of well defined bands in this blot show that this method of probing with lectins has potential. The bands in S_2, with the longer run time, are generally sharper than in S_1.

Blot 2 is also visualised using Con A, although the stain has not 'taken' as well. However, banding in the alkaline region can be seen in sample B which is positive for oligoclonal IgG. The samples were run in duplicate and, in Blot 3, visualised using SNA. This lectin, which has a binding preference for sialic acid is seen to bind only in the acidic half of the gel strips.

Blot 4 shows an oligoclonal positive CSF (O) with two negatives (M and H), with Con A detection. There are very pronounced bands in the alkaline region for CSF O. This CSF was run in duplicate and also visualised using SNA (Blot 5), however with this lectin, there is no staining in the alkaline region.

5.4.2. Results for Isoelectric Focusing with pH 7 - 10 Gradient

Figure 5.4 shows the PVDF blot of an electrofocused CSF with fractions from a Prosep column. The low concentrations of IgG in the eluted fractions E_1 and E_2 have resulted in attenuated patterns of banding. However, the main oligoclonal IgG bands in the whole CSF appear to be replicated in the eluates, suggesting that the oligoclonal IgG is not affected by the low pH of the elution buffer, and that no IgG isoforms are lost in the

affinity chromatography process. There is no difference between E_1 and E_2, using different volumes of elution buffer. There is faint staining in W, the wash fraction from the column. This may be from traces of IgG which are not bound by the protein A, or may be carryover from the CSF sample during application of samples to the gel.

Figure 5.5 shows the results of the first deglycosylation study. In the anti-IgG detected blot, oligoclonal IgG bands are clearly seen in the whole CSF (CE). The banding pattern is replicated in the non-deglycosylated eluate (D). In the deglycosylated eluate (C), again there are well-defined bands, but the pattern of banding is altogether different to CE and D. In the denatured samples (A and B), there is virtually no staining, indicating the treatment has either rendered the IgG so that it is not detected by the stain, or has changed the charge of the IgG so that it has migrated out of the pH range. In the Con A detected blot, bands in the whole CSF (CE) are again replicated, albeit fainter, in the non-deglycosylated eluate (D). However, in the deglycosylated sample (C) there is hardly any staining visible, indicating, within the detection limits of the system, that the PNGase F treatment successfully removed all Con A binding glycans from the IgG. As all IgG glycans contain mannose, to which Con A binds, deglycosylation of the IgG is complete. The results of the second deglycosylation study are shown in Figure 5.6. Again, in the anti-IgG detected blot, bands in the non-deglycosylated eluate (NPB) correspond to bands in the whole CSF (NP), whereas in the enzyme-treated eluate (NPA) the banding pattern has changed. In the Con A detected blot, the non-deglycosylated eluate (NPB) has a similar pattern to the CSF (NP), while there is faint banding in the enzyme treated eluate (NPA), indicating that there may be some glycans still remaining on the IgG. There is similar faint banding in the enzyme blanks (E), corresponding to heavy bands in the CSF, and is thought to be due to carryover from other samples, rather than due to the enzyme itself.

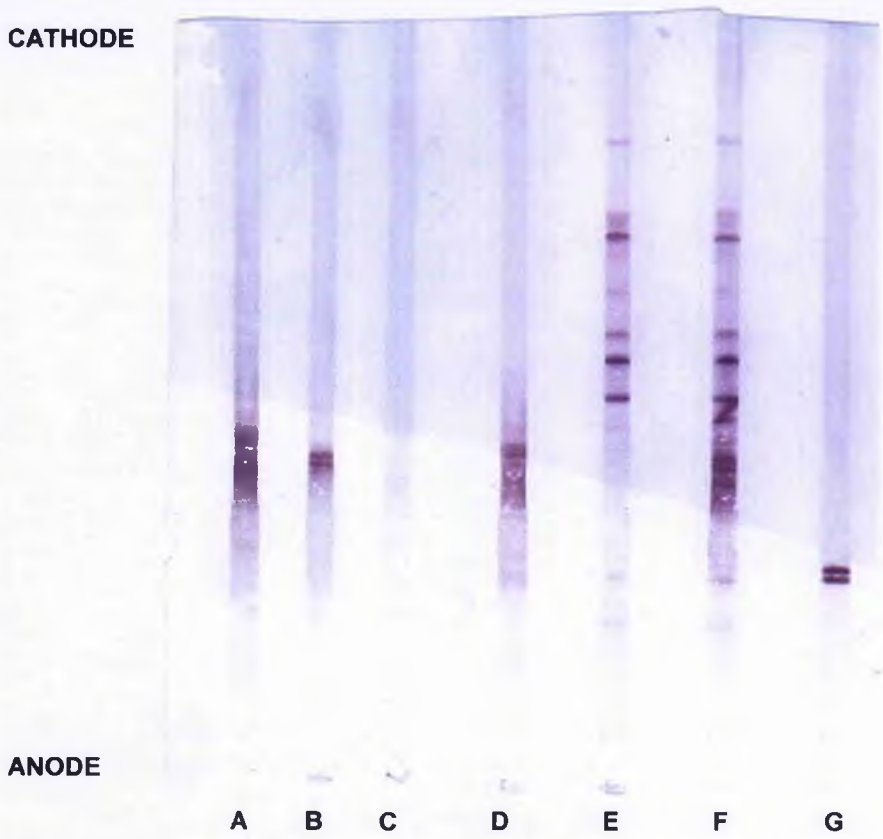


Figure 5.1. PVDF blot of gradient pH 3 –10 gel strips, run at 300 V for 1 h then 1400 V for 15 h. Detection was with colloidal gold stain. Samples applied were 2 μL of:- **A**: eluate of CSF M from a protein A column with 1500 mg/L BSA added; **B**: the eluate with 150 mg/L BSA; **C**: the eluate with no BSA; **D**: 0.5 M phosphate buffer with added 1500 mg/L BSA; **E**: broad pI calibrators; **F**: broad pI calibrators with added 1500 mg/L BSA; **G**: β-Lactoglobulin.

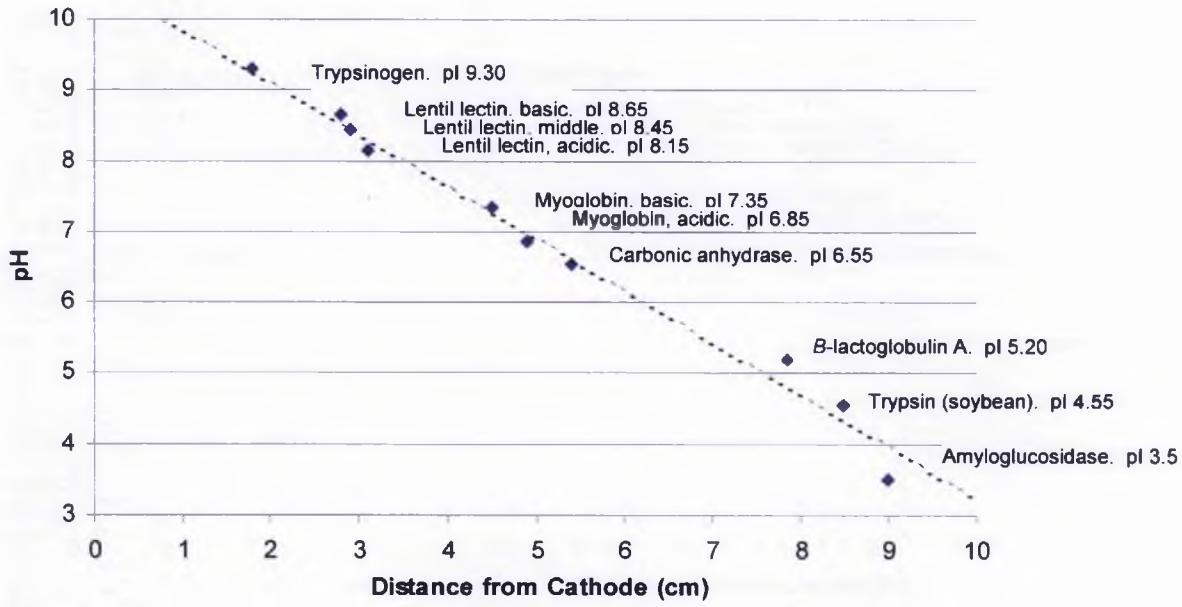


Figure 5.2. Distance from cathode of bands from broad pI calibrators (sample E in Figure 5.1) plotted against pH. The identities of the bands were confirmed by additional runs of the broad calibrator alongside single purified proteins.

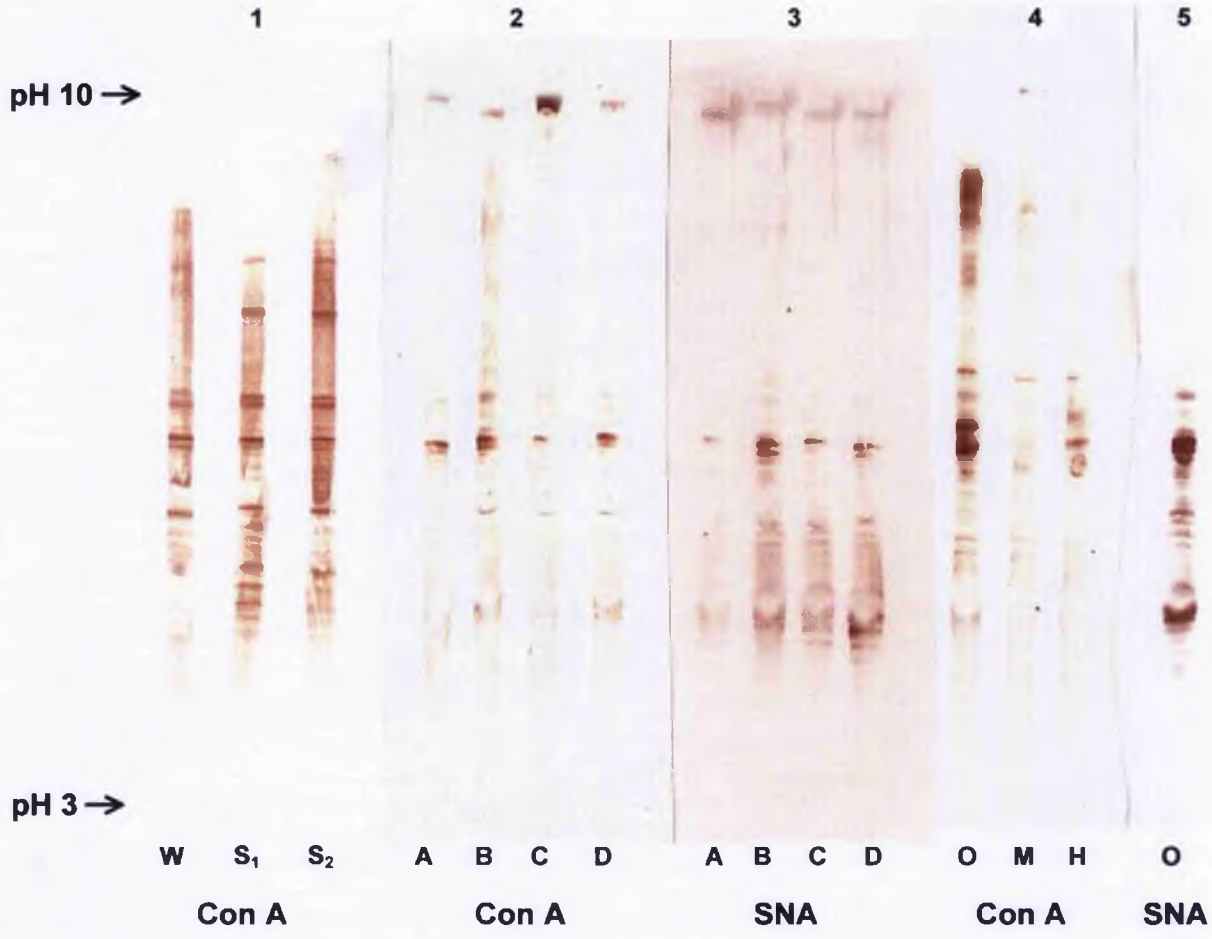


Figure 5.3. A selection of five PVDF blots of CSF samples run on pH 3 - 10 gel strips. Apart from W and S_1, which were run at 300 V for 1 h then 1400 V for 6 h, all samples were run at 300 V for 1 h then 1400 V for 15 h. (S_1 and S_2 are the same sample, but with different run conditions). 2 μL samples were applied onto the centres of the gel strips. Detection was achieved using the biotinylated lectins as shown, with avidin-HRP and DAB.

Blot 1 shows CSF W, which was negative for oligoclonal IgG bands by routine IEF, and CSF S, which was positive. Blots 2 and 3 are of four CSFs run in duplicate, with detection using Con A and SNA respectively. The CSFs A, C and D were negative for oligoclonal bands, while B was positive. Blot 4 is of the oligoclonal positive CSF O and the oligoclonal negative CSFs M and H, detected using Con A. Blot 5 is of CSF O, run by IEF simultaneously as Blot 4, but detected using SNA.

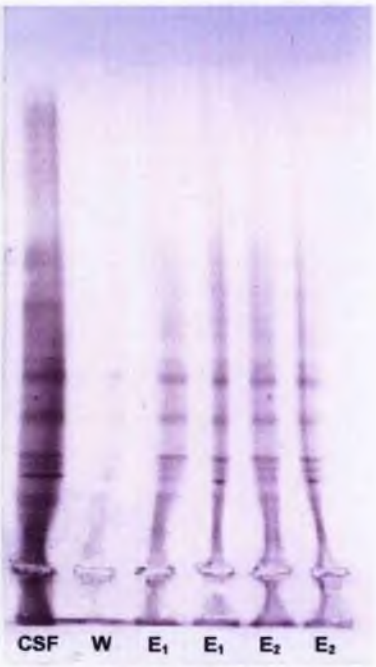


Figure 5.4. PVDF blot of a CSF containing oligoclonal IgG bands and fractions from a Prosep A column run on pH 7 to 10 IEF gel, with detection using sheep anti-human IgG and donkey anti-sheep alkaline phosphatase conjugate. IEF and staining was kindly performed by Dr G. Cowdrey, as described in sections 5.3.2 and 5.3.3.3. Affinity chromatography using a 0.2 mL Prosep A column was as described in 4.5.3.1(a).
'CSF' is the untreated CSF sample; 'W' is the breakthrough collected on washing the column; 'E_1' is the eluate collected onto neutralising buffer, and applied to the gel in duplicate. 'E_2' is the eluate collected with modification of the chromatography procedure i.e. using half the volume of elution buffer.

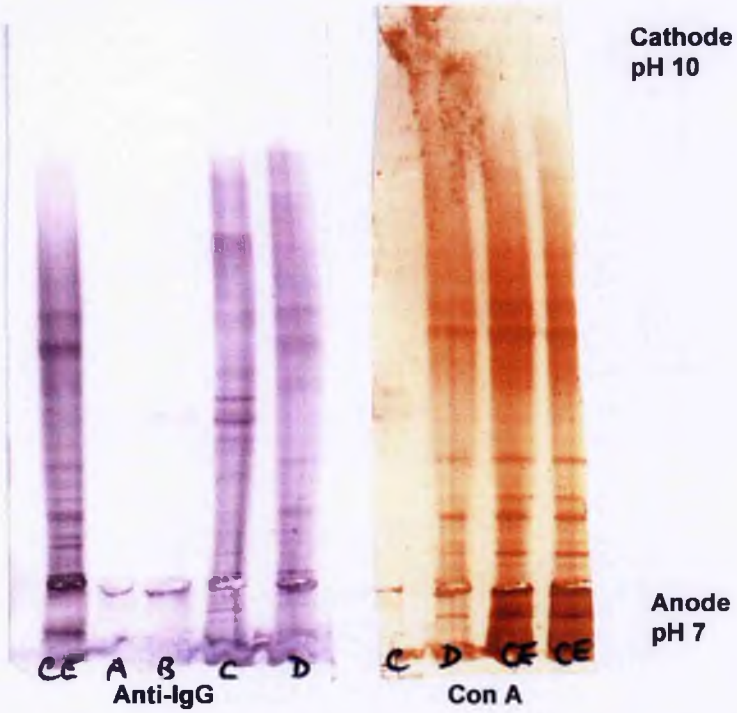


Figure 5.5. PVDF blots of isoelectric focused CSF IgG before and after deglycosylation with PNGase F. Samples were run simultaneously on a pH 7 to 10 gel by Dr G. Cowdrey as described in 5.3.2. Detection was with sheep anti-human IgG/anti-sheep alkaline phosphatase conjugate (see section 5.3.3.3) and with biotinylated Con A/HRP (section 5.3.3.1).

CE is the untreated CSF (containing oligoclonal IgG bands). A, B, C and D are portions of the IgG-containing fraction from the same CSF eluted from a Prosep A column. A and B were treated with denaturing agents; C and D were not. A and C were treated with PNGase F; B and D were not. For method details see 5.3.5.1.

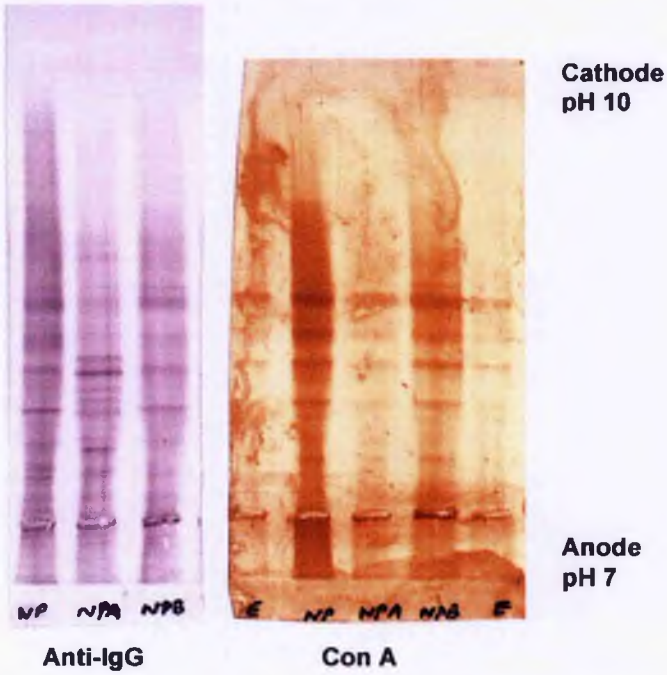


Figure 5.6. PVDF blots produced as for Figure 5.5 for a different oligoclonal IgG positive sample (CSF NP), with some changes in the procedure i.e. denaturation of a portion of the sample was omitted, double the quantity of enzyme was used and an enzyme blank was run. For full method details see 5.3.5.2.

NP is the untreated CSF; NPA is eluate treated with PNGase F; NPB is untreated eluate. E is buffer containing PNGase F only.

5.5. Discussion

Much work could still be done using IEF to investigate the glycosylation of IgG. The practical work using the pH 3 - 10 gel strips is largely incomplete. The IEF technique and detection using lectins was very time-consuming and required a high degree of manipulative skill, which would probably come with practice. But, with time at a premium, the pH 7 - 10 gel system was used instead for the deglycosylation study. Nevertheless, the high resolution of the bands on some of the PVDF blots of the pH 3 - 10 gel strips illustrate the potential that the method has.

The concentration of IgG in the Prosep protein A eluates prepared for the lectin immunoassay was too low for detection with the lectin assays. Increasing the sample applied to the gel led to problems with overheating, because of focusing of the salts in the eluate into high concentration bands. However, for the deglycosylation studies, the use of dialysis to remove salts from the eluates enabled affinity purified IgG to be run by IEF. Also, the BSA hitherto used to stabilise the IgG in the eluates, showed numerous bands on the PVDF blots with detection by colloidal gold. It would be preferable then to use highly purified BSA or HSA (human serum albumin), or a non-protein product as stabiliser, or to perform the affinity chromatography immediately before the IEF.

With the detection using lectins, Con A - which binds to mannose, present in all *N*-linked glycans - is seen to bind to oligoclonal bands throughout the pH 3 to 10 range. SNA, however, binding only to glycans containing sialic acid, shows binding in the acidic and neutral range, but not to oligoclonal IgG bands in the alkaline region. These findings are compatible with the hypothesis that oligoclonal IgG bands in the alkaline region are lacking sialic acid, although with so few samples it is impossible to be conclusive.

With enzymic deglycosylation, prior denaturation of the target protein is often performed. However, in this study almost total deglycosylation of the IgG was achieved without denaturation, as illustrated by the absence of Con A binding to the deglycosylated

samples (Figures 5.5 and 5.6). It is feasible that deeply buried glycosylation, for example in the $C_\gamma 2$ domains, is not reached by the enzyme nor by Con A. But if glycosylation is complete, the altered pattern of bands in the deglycosylated sample compared with the untreated sample shows that the spread of isoelectric points of oligoclonal IgG bands is not simply due to differences in carbohydrate. Were this the case, the oligoclonal IgG bands would coalesce into a single, or only a few, bands. As it is, the resultant bands in the deglycosylated sample are as diverse as those in the untreated sample, but with a different pattern.

Chapter Six

Analysis of IgG Glycans by HPLC

6.1. Introduction

The results of the lectin immunoassays suggest there is a difference between MS patients and non-MS controls in the glycosylation of CSF IgG. To try and show the difference, HPLC was used to analyse the *N*-glycans after cleavage from IgG by hydrazinolysis and labelling with 2-aminobenzamide (2AB). A normal phase HPLC system was used for all samples, which separates largely by the hydrodynamic volume (size) of the glycan and to a lesser extent by the hydrophilicity of individual sugars and antenna linkage. Some samples were also run on a weak anion-exchange column, separating by overall negative charge, which would show differences in sialylation.

The purification of IgG by protein A column chromatography and subsequent dialysis and spectrophotometry was carried out at the Princess Royal Hospital Biochemistry Department. The lyophilisation and glycan release was carried out by Dr Louise Royle at the Glycobiology Institute, Oxford University. The labelling and HPLC was carried out mostly by myself at the Glycobiology Institute, using the systems set up in those laboratories, and relying on help and guidance from the staff there.

6.2. Equipment and Reagents

Reagents for protein A affinity chromatography are as described in section 4.6.2.

1. Slide-A-Lyzer dialysis cartridge. 3500 molecular weight cut-off; 0.1 to 0.5 mL capacity. Product No. 66335. Pierce Chemical Company, Rockford, IL 61105, USA.

2. Scanning spectrophotometer. U-2000. Hitachi Ltd., Tokyo, Japan.
3. GlycoPrep 1000 for automated release and purification of glycans. Oxford GlycoSystems, Blacklands Way, Abingdon, OX14 1RG.
4. Centrifugal sample evaporator. Eyela Vapour Mix S-100. Tokyo Rikakikai Co Ltd. (Eyela-ex@eyela.co.jp).
5. Speed sample evaporator. GyroVap. V.A. Howe Ltd., Banbury, U.K.
6. Chromatography paper. 3M. Whatman Ltd., Maidstone, England. Prior to use this was washed with water by descending chromatography for 24 h.
7. HPLC system. 2690 HPLC Separations Module with column heater; 474 fluorescent detector; Millennium 32 software. Waters Corp. 34 Maple St., Milford, Massachusetts 01757, USA.
8. HPLC columns 1) GlycoSep-N normal phase, size 4.6 x 250 mm (Oxford GlycoSystems). 2) Protein WAX: weak anion-exchange, size 7.5 x 50 mm (Vydak, Crompack, Middelburg, Netherlands).
9. Trifluoroacetic acid (TFA). Biochemika grade, >99.5% purity. Product No 91699. Fluka/Sigma Co.
10. 2-Aminobenzamide (2AB). Signal kit. K-404. Oxford GlycoSystems.
11. Acetonitrile, methanol. HPLC grade from Romil Ltd., The Source, Convent Drive, Waterbeach, Cambridge, CB5 9QT.
12. Ammonia solution, formic acid. Aristar grade from Merck Ltd.
13. Solutions of 50 mM ammonium formate, pH 4.4 and 0.5 M, pH 9.9 were prepared by adjusting formic acid solution to the required pH using ammonia solution and diluting to give the appropriate concentration.
14. HPLC calibrators. System 1: Dextran homopolymer standard, lyophilised 7.5 mg. Product code I-4014. System 2: Fetuin (bovine) glycans I-4015, lyophilised 5 mg. Product code I-4015. Oxford GlycoSystems.

6.3. Samples

A relatively large amount of IgG was required in the CSF samples, owing to losses that occur during the lengthy preparation prior to HPLC. The initial recommendation from the Oxford Glycobiology Institute was for at least 50 μg of IgG in the samples ready for labelling, although this was decreased to 20 μg for the second batch of samples. This meant that only a small proportion of the CSFs after routine analysis were suitable, and it was not possible to select samples for HPLC by the same criteria as for the lectin immunoassays, where a confirmed diagnosis of MS was required for the positive group (see section 4.6.3). This will have important implications when comparing the results of the two methods.

The samples for HPLC were collected over a period of one year. In view of the lengthy preparation procedure and the high cost of reagents, an initial batch of 4 samples was analysed. The encouraging results from this batch led to analysis of a further batch of 10 samples.

The criteria for selecting Positive samples were:

1. Strong oligoclonal IgG bands on IEF.
2. At least 50 μg of IgG in the sample (at least 100 μg for first batch).

The criteria for selecting Negative Control samples were:

1. Absence of IgG oligoclonal bands on IEF.
2. At least 50 μg of IgG in the sample (at least 100 μg for first batch).

Details of the samples assayed by HPLC are given in Table 6.1. To help distinguish the samples, the samples containing IgG oligoclonal bands are identified by upper case lettering, while the negative controls are in lower case.

6.4. Methods

6.4.1. Affinity Chromatography

1. The protein A columns were prepared as described in Section 4.5.
2. The amount of each sample applied to the column, and the IgG content, is shown in Table 6.1. Each sample was diluted with an equal volume of adsorption/wash buffer before applying to the column. The (RH) serum sample was diluted with 1.0 mL of buffer.
3. When the sample/buffer mixture had drained through, a further 1.0 mL of adsorption/wash buffer was applied and allowed to drain.
4. 0.1 mL of elution buffer was applied and allowed to drain to waste.
5. 0.5 mL of elution buffer was applied and the eluate collected onto 0.1 mL of neutralising buffer in a glass bottle.
6. The eluates were immediately dialysed.

6.4.2. Dialysis

1. The Slide-A-Lyzer cassettes were used according to the manufacturer's instructions. Each eluate (volumes of eluates were approximately 0.6 mL) was injected into a cassette and dialysed against 1.0 L of deionized water containing 0.7 mL of TFA for 24 h, changing the dialysis solution twice.
2. The dialysate was removed from the cassette and placed into a 3 mL lyophilisation tube. 0.2 mL of water was injected into the cassette, which was agitated for 20 s, then removed and added to the dialysate.
3. 100 μL of the dialysate was placed into a quartz glass cuvette for spectrophotometry, as described below, then returned to the lyophilisation tube, which was then stored at -70°C.

Table 6.1. Details of samples processed for HPLC.

	Sample	Oligo-clonal Bands	CSF Total Protein (TP) mg/L	CSF IgG mg/L	IgG % TP	Clinical Details, MRI scan	Applied to Column Vol. (mL)	IgG (μg)
	DH	Positive	437	69	15.7	Probable MS.	1.7	117
First	GH	Positive	950	112	11.7	1° progressive MS.	1.3	146
Batch	ms	Negative	568	39	6.9	Meningioma.	2.7	105
	ja	Negative	482	44	9.1	No diagnosis of MS or inflammatory disease.	2.5	110
	AW	Positive	518	43	8.3	MRI scan normal; presumed ocular sarcoidosis.	3.0	129
	HC	Positive	464	177	38.1	MRI scan normal; haematoma.	0.5	88
	BP	Positive	226	31	13.7	MRI scan not done; possible 1° progressive MS.	1.7	53
Second	AR	Positive	676	189	27.9	MRI scan suggests demyelination; sensory disturbance.	1.0	189
Batch	RH	Positive	624	65	10.4	MRI scan suggests demyelination; possible 1° progressive MS.	1.2	78
	RH (serum). IgG = 10.6 g/L						0.010	106
	dg	Negative	280	25	8.9	MRI scan and history strongly indicate MS.	1.9	48
	ib	Negative	263	34	12.9	Cerebral palsy; MRI scan suggests possible demyelination	2.4	82
	jt	Negative	231	22	9.5	MRI normal; ?familial spastic paraplegia	2.1	45
	al	Negative	515	38	7.3	Bilateral papilloedema; MRI scan not done.	2.4	91

6.4.3. Spectrophotometry

Purified human IgG was diluted to 5 mg/L and 100 mg/L in dialysis fluid (1.0 L deionized water containing 0.7 mL TFA). These solutions, with the dialysate for the sample AW, were scanned in quartz cuvettes against air between 200 and 360 nm. The scans were used to ascertain the best wavelength for measurement of the IgG in the dialysates.

Calibration solutions were prepared as follows. 50 μL of the human IgG was diluted in 2.8 mL of dialysis fluid to produce a 100 mg/L solution. This was then further diluted in dialysis fluid to give 80, 60, 40, 20, 10 and 5 mg/L solutions. The absorbance of these was measured at the selected wavelength against dialysis fluid, using quartz cuvettes, and a calibration graph was constructed. Dialysates were measured similarly, and the [IgG] determined from the calibration graph. The IgG content of the dialysate was then calculated.

6.4.4. Lyophilization

The samples were posted, frozen, to Oxford. There they were thawed out, centrifuged, then lyophilised over 2 days - this work carried out by Dr Louise Royle.

6.4.5. Glycan Release

The glycans were released from the IgG and purified by Dr Royle, using the GlycoPrep 1000 in N* mode for *N*-glycans. The GlycoPrep uses hydrazine to release glycans in a quantitative manner, followed by column chromatography to remove protein/peptide material. Samples were processed in pairs, running each pair overnight.

6.4.6. Labelling with 2-Aminobenzamide (2AB)

Double distilled water was used throughout. The samples from the GlycoPrep were dried using the GyroVap (approximately 2 h). The 2AB Signal kit was used according to the manufacturer's instructions: 5 μL of the prepared labelling mix was added to the dried samples and to the dextran and fetuin calibrators, followed by vortex

mixing, centrifuging and incubating for 2 h at 65°C. (The samples were re-vortexed and centrifuged after 15 min to ensure that any slow-dissolving material was mixed.).

The labelled samples were cleaned up as follows. Washed chromatography paper was cut into strips using a template. After incubation, the samples were spotted onto the paper strips and dried for 2 h. The strips were inserted into Teflon holders, stood in acetonitrile and subjected to ascending chromatography for 1 hour. Labelled glycans remain on the origin: unbound 2AB runs up the paper. Completion was checked by visualising under a UV lamp. After drying the paper, the spots containing labelled glycans were cut out under the UV lamp.

Elution of labelled glycans was performed as follows. The paper cut-outs were placed into marked 1 mL polypropylene syringes, with PTFE filters fitted to the nozzle. The syringes were positioned over glass Rotovap tubes (previously soaked overnight in 4M HNO_3 then washed 10 times in deionized water) and 1 mL of water placed inside. After 10 min, the liquid was pushed through the syringes. This was repeated 3 more times, but adding 0.5 mL of water each time.

The labelled glycan solutions were concentrated prior to HPLC as follows. The tubes were placed on the Rotovap, with vacuum suctioning and vortexing, and an infrared lamp warming the outlet to prevent condensation of the evaporated liquid. After evaporation to dryness, 1.0 mL of water was added to each tube, with vortexing and centrifuging, then rotary evaporated again to dryness. This was repeated with 0.5 mL of water. The purpose of the reduction of liquid is to obviate loss of glycan material on the sides of the tubes. To each tube was then added 0.25 mL of water, followed by vortexing, centrifuging, and pipetting of the water into a marked polypropylene Eppendorf micro-tube, and this procedure repeated with 3 more 0.25 mL additions of water. Finally, the samples were evaporated to dryness (GyroVap) and 100 μL of water (for first batch) or 200 μL of water (for second batch) was added and vortex mixed.

6.4.7. HPLC

6.4.7.1. System 1. GlycoSep N Column

Running conditions are shown in Table 6.2. The injection volume was 95 µL. For the first batch, 1 µL of each reconstituted labelled sample or calibrator was placed into a HPLC vial with 19 µL of double distilled water and 80 µL acetonitrile (i.e. 1% [^{v}/v] sample dilution). For the second batch, 4 µL of sample plus 16 µL of double distilled water and 80 µL of acetonitrile (i.e. 2% [^{v}/v] sample dilution) was run initially for some samples to obtain an idea of the dilutions needed to be within the detector range. All samples were then diluted appropriately, according to Table 6.3.

6.4.7.2. System 2. Vydac Protein WAX Column

Samples only from the first batch, i.e. ms, ja, GH and DH, were run on this system, processed as described in 6.4. The HPLC work was carried out by Dr Royle at the Institute of Glycobiology, Oxford. 5 µL of each reconstituted, labelled sample or labelled calibrator was pipetted into an HPLC vial with 95 µL of double distilled water. The running conditions are shown in Table 6.4.

Table 6.2. Running conditions for HPLC System 1.

	Time (min)	Flow (mL/min)	%A	%B
Equilibration	180	0.4	20	80
Injection	0	0.4	20	80
	152	0.4	58	42
	155	0.4	100	0
	163	1.0	20	80
	178.5	0.4	20	80

A is 50 mM ammonium formate, pH 4.4; B is acetonitrile.
The column was re-equilibrated in 20% A, 80% B before injection of each sample. Total run time for a sample was 180 min. The column temperature was set at 30°C. The detector was set at 330 nm excitation wavelength, 420 nm emission wavelength. Sample injection volume was 95 µL.

Table 6.3. Dilution of labelled samples for HPLC System 1.

Sample	Recon-stitution volume (μL)	Sample volume (μL)	Water volume (μL)	Acetonitrile volume (μL)	Sample Dilution % (^{v}/v)
ms, ja, GH, DH	100	1	19	80	1
AR, al	200	2	18	80	1
HC	200	4	16	80	2
AW, BP, dg, ib, RH(serum), jt	200	20	0	80	10
RH	80	20	0	80	25

6.5. Results

6.5.1. Spectrophotometry

Figure 6.1 shows the spectrophotometric scans of (A) diluted IgG, (B) dialysis buffer and (C) a dialysate from affinity purified CSF sample. From these scans (and other scans of IgG solutions) an absorbance of 279 nm was chosen for the quantitation of IgG in the dialysates. Figure 6.2 gives the calibration plot for measuring IgG in the dialysates. The IgG content of the dialysates is given in Table 6.5.

Table 6.4. Running conditions for HPLC System 2.

	Time (min)	Flow (mL/min)	%C	%D
Equilibration	180	1.0	100	0
Injection	0	1.0	100	0
	12	1.0	95	5
	25	1.0	21	79
	50	1.0	20	80
	55	1.0	0	100
	66	1.0	100	0
	80	1.01	100	0

C is 0.5 M ammonium formate, pH 9.0; D is 10 % (^{v}/v) methanol in water.
The column was re-equilibrated in 100% C before injection of each sample. Total run time for a sample was 80 min. The column temperature was set at 30°C. The detector was set at 330 nm excitation wavelength, 420 nm emission wavelength. Sample injection volume was 95 μL.

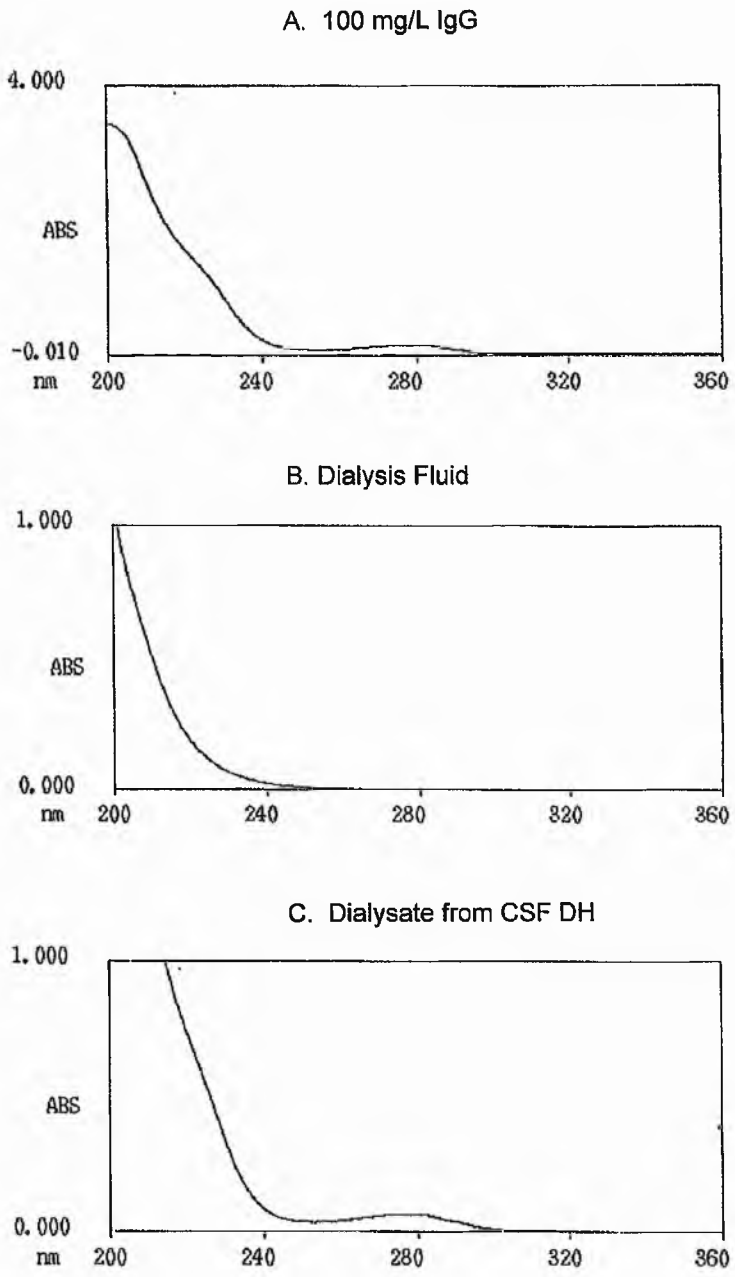


Figure 6.1. Spectrophotometric scans of: A: pure human IgG, 100 mg/L in dialysis fluid; B: dialysis fluid; C: The dialysate from CSF DH.

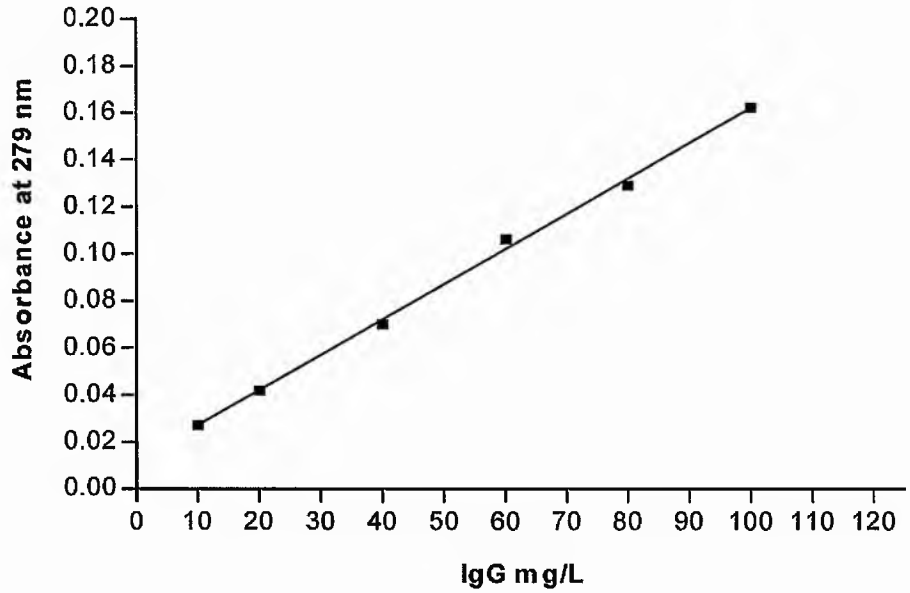


Figure 6.2. Calibration plot for measuring the IgG content of dialysates. Solutions containing less than 10 mg/L of IgG were also measured, but it was found linearity did not extend below this level. The correlation coefficient (r) for the fitted graph is 0.999 (y = 668.7x + 0.01207). The formula: $[IgG]_{mg/L} = (A_{279\ nm} - 0.012) \times 668.7$ was used to obtain the IgG concentration of the dialysates.

Table 6.5. Details of samples for HPLC showing IgG content of dialysates.

	Sample	Oligoclonal Bands	CSF IgG mg/L	Dialysate IgG µg
	DH	Positive	69	42
First	GH	Positive	112	82
Batch	ms	Negative	39	62
	ja	Negative	44	68
	AW	Positive	43	30
	HC	Positive	177	56
	BP	Positive	31	30
Second	AR	Positive	189	82
Batch	RH	Positive	65	10*
	RH (serum; [IgG] = 10.6 g/L			57
	dg	Negative	25	20
	ib	Negative	34	42
	jt	Negative	22	28
	al	Negative	38	55

*Small volume of dialysate due to spillage during processing.

6.5.2. HPLC

6.5.2.1. System 1. GlycoSep N Column

a. First Batch

Figure 6.3 shows the chromatograms of the first batch of samples. The overall patterns of the chromatograms is similar to patterns for serum IgG found previously (e.g. Guile et al, 1996) (Figure 1.10). The calibrator, partially hydrolysed dextran, was used to assign GU values to retention times, enabling peaks to be identified by comparing the GU values to the examples of Guile et al, and by using tables of GU values compiled by the Oxford Glycobiology Institute for the same HPLC system. Figure 6.4 shows the plot of the retention time of the dextran polymers against their component glucose units, which is seen to be curvilinear. Figure 6.5 shows the structures assigned to the peaks for sample ja: the other samples were processed similarly.

The two negative control samples have a very similar pattern and closely resemble the pattern (Figure 1.10b) found by Guile et al (1966) for serum IgG from RA patients. They have the distinctive large peak containing fucosylated agalactosyl biantennary glycan (A2G0F) at 85 min. The triplet of peaks, with the retention times of 92 and 95 min, is very similar for both control samples.

The two positive samples, however, differ from each other and from the negative samples and generally show wider variations than the controls. The triplet peaks have different patterns, and of the four samples, DH has the largest agalactosyl (A2G0) peaks, between 81 and 89 min, while GH has the largest digalactosyl (A2G2) peaks, between 100 and 112 min.

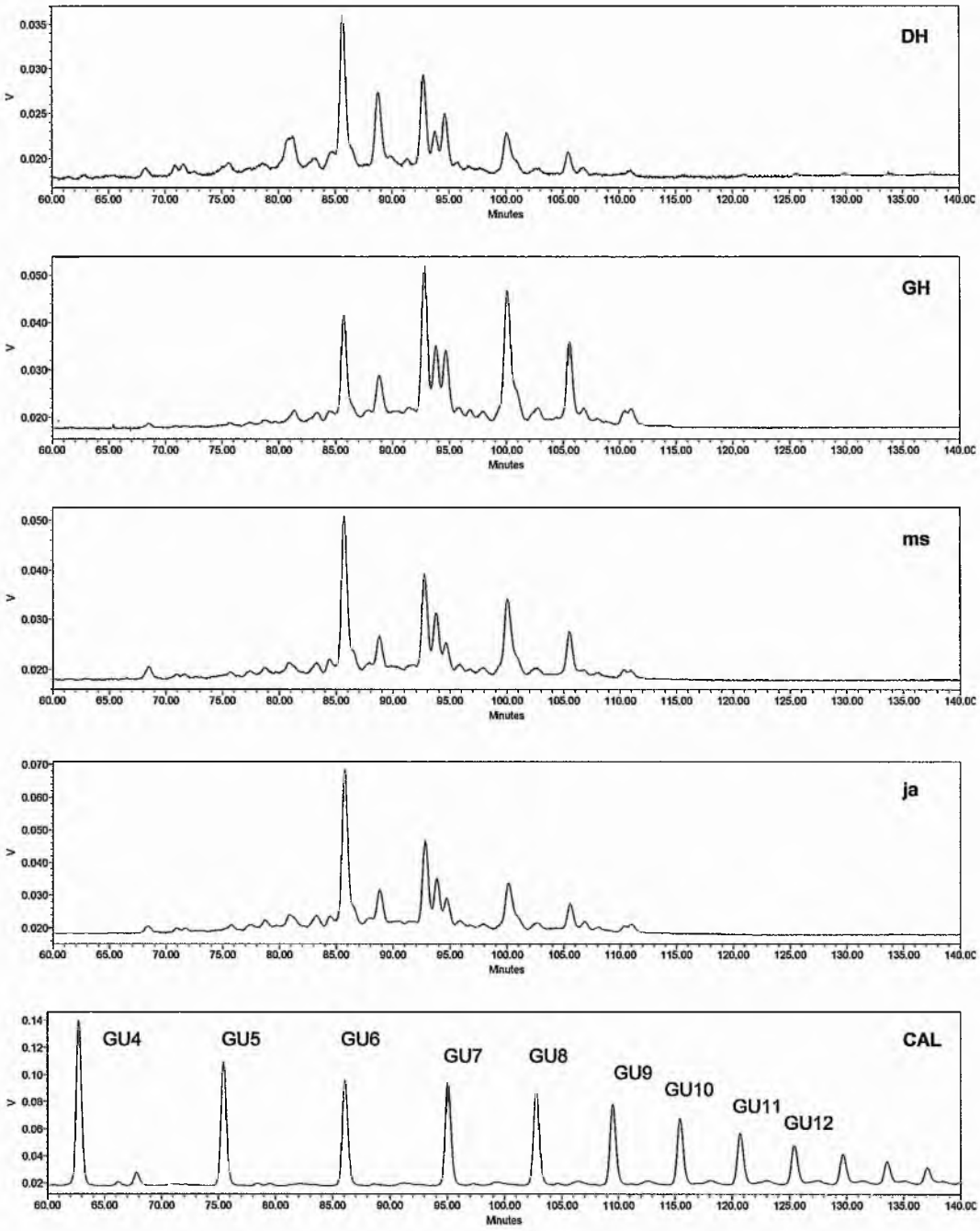


Figure 6.3. Chromatograms of the first batch of samples, labelled with 2AB, run by normal phase HPLC (System 1). For running conditions, see 6.4.7.1.
DH and GH are CSF samples containing oligoclonal IgG; ms and ja are oligoclonal IgG negative samples. (In this and the following figures, upper case letters are used to denote positive oligoclonal IgG samples, and lower case negative controls).
CAL is the calibrator, containing homopolymers of glucose derived by partial hydrolysis of dextran. Thus GU4 contains four glucose units, up to GU12 containing twelve glucose units.

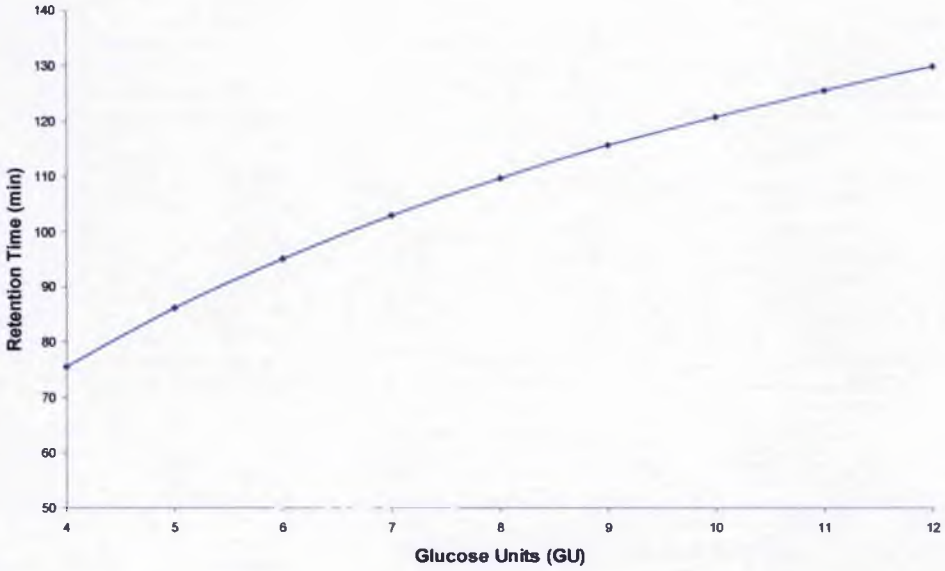


Figure 6.4. GU calibration of HPLC System 1. The number of glucose units (i.e. GU value) in each dextran polymer is plotted against the retention time. The curve is fitted by 5th order polynomial regression. The retention times of the sample peaks can then be assigned GU values to enable identification of the glycan structures comprising the peaks.

b. Second Batch

Chromatograms for the second batch of CSF samples are shown in Figures 6.6 (negative controls) and 6.7 (oligoclonal IgG positive samples). Figure 6.7 also shows the serum sample of RH with the matching CSF. The peaks in both sets are not as well resolved as those in the first batch, especially noticeable with the triplet of peaks at 92 to 95 minutes. Nevertheless, assignment of glycan structures to the peaks as performed for the Batch 1 samples was not a problem, although quantitation of peak areas was probably not as accurate.

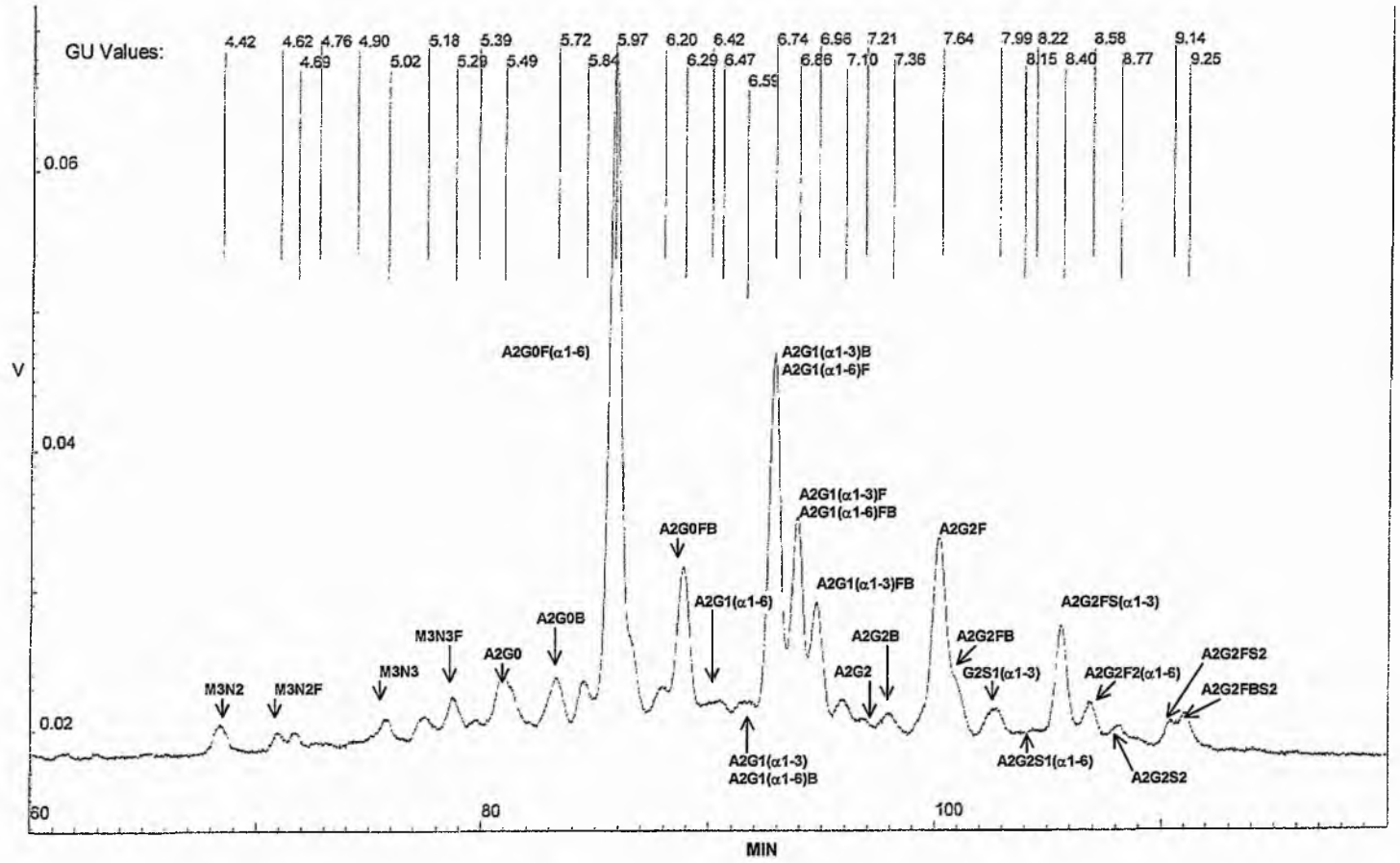


FIGURE 6.5. Chromatograph of the sample ja, showing the GU values of the peaks, calculated by the HPLC software by comparison with the dextran calibrator, and printed as shown. Structures were assigned to the peaks where possible by referring to previous chromatograms (Guile et al, 1996) and tables of GU values compiled by the Oxford Glycobiology Institute. A2 indicates biantennary glycans; G (0, 1 or 2) indicates number of galactose residues; S (0, 1 or 2) indicates number of sialic acids; (α1-3) or (α1-6) indicates type of glycosidic linkage; F denotes the presence of fucose, and B denotes a bisecting GlcNAc residue. M and N indicate numbers of mannose or GlcNAc residues respectively in core products. Structures were assigned to the peaks in the same way for the other samples.

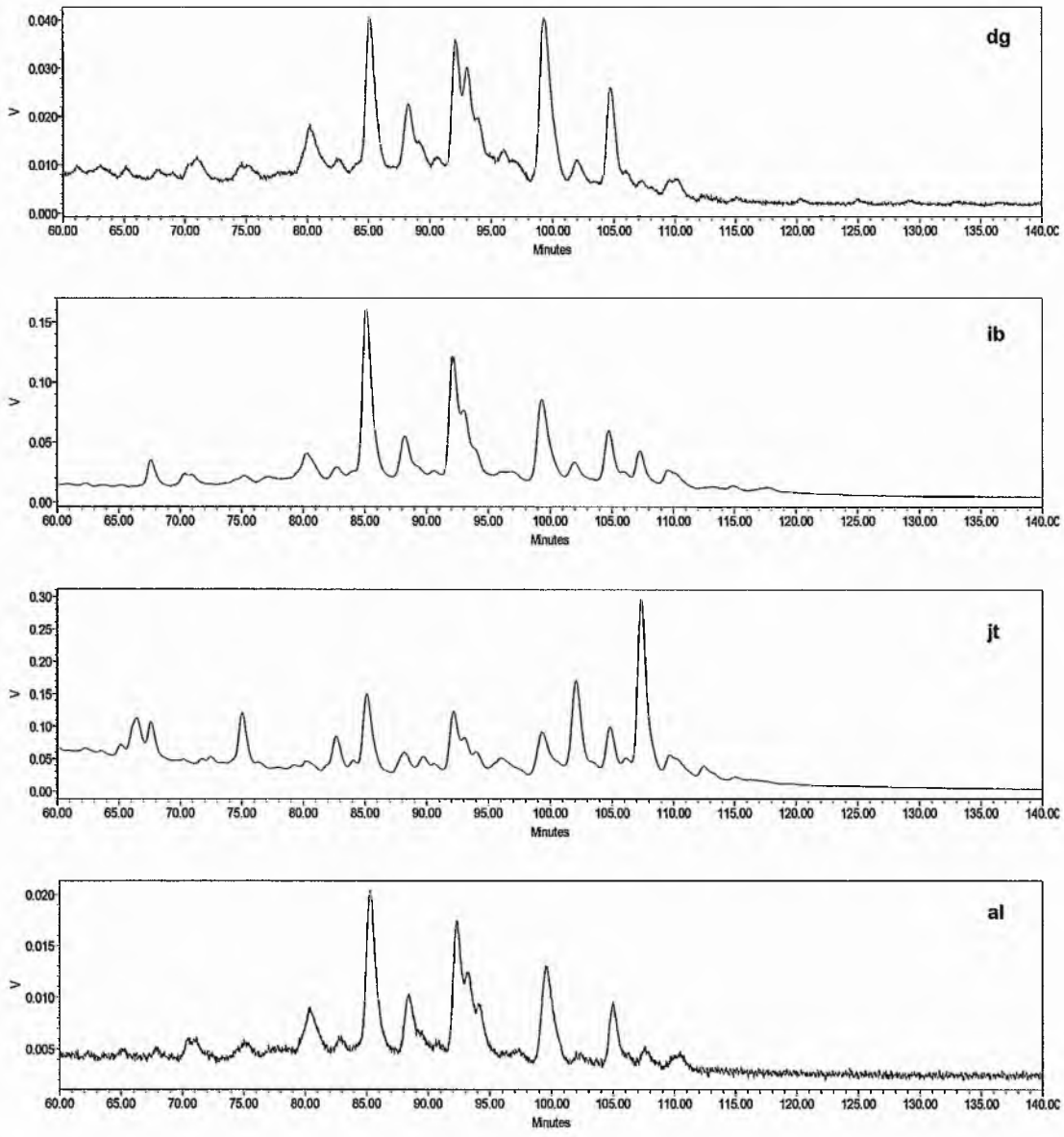


Figure 6.6. Chromatograms of the negative control samples in the second batch run on normal phase HPLC (system 1) after labelling with 2AB. For method details see section 6.4.7.1.

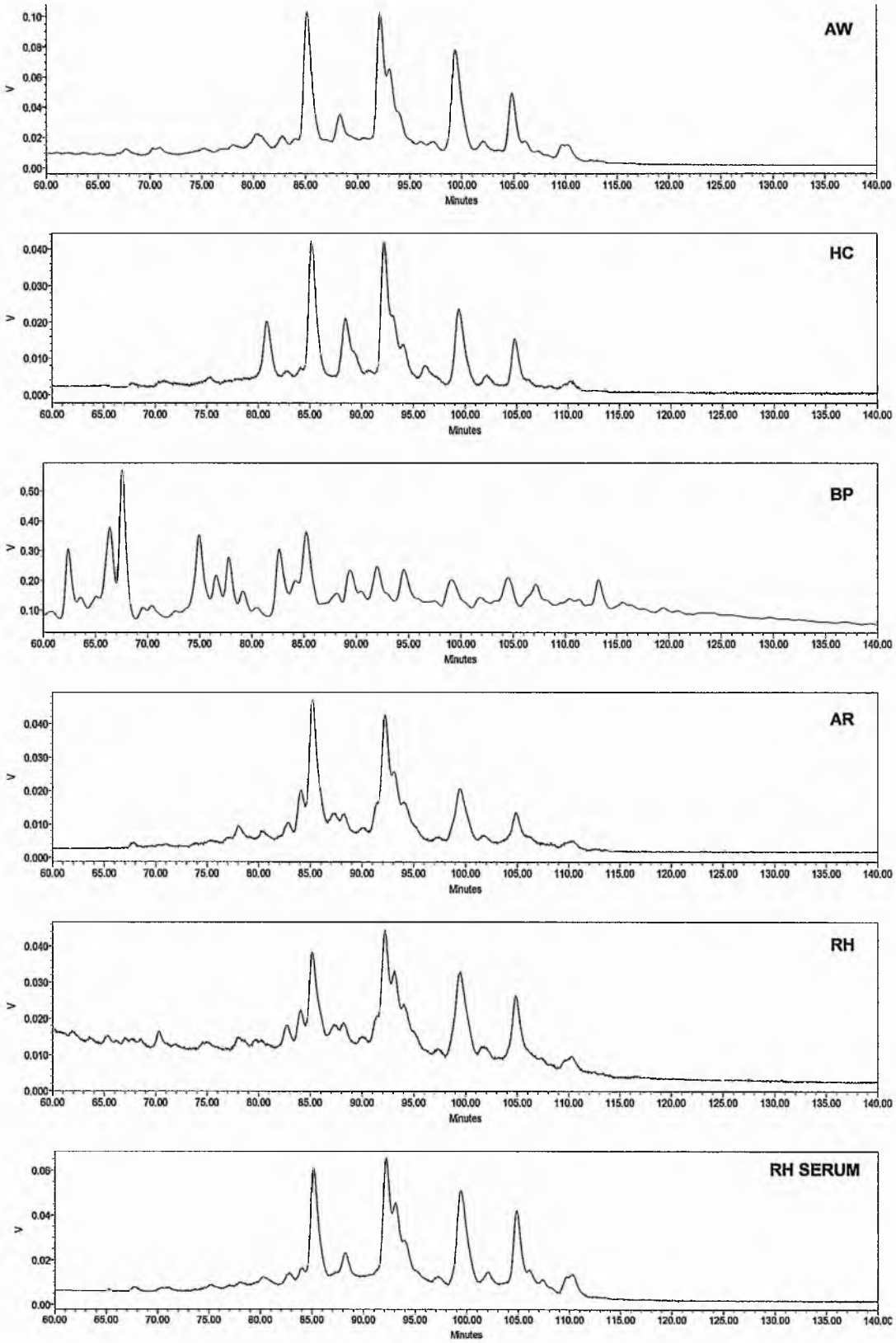


Figure 6.7. Chromatograms of the oligoclonal IgG positive samples in the second batch run on normal phase HPLC (system 1) after labelling with 2AB. 'RH serum' is a serum sample paired with the CSF 'RH'.

Unlike the first batch, there is some dissimilarity between the negative samples. However, the size of the A2G0F peaks at 85 min and the appearance of the triplet peaks at 92-95 min are similar in the four samples. Other major peaks at 80 min (A2G0), 89 min (A2G0FB), 100 min (A2G2F) and 105 min (A2G2FS) vary considerably in peak height. The sample jt appears to be highly sialylated with exceptionally large peaks at 102 min (A2G2S1) and especially 108 min (A2G2S2), which dominates the chromatogram. This sample also has some relatively large peaks between 60 and 80 min i.e. GU values between 4 and 5.5. The structures with GU values in this region are core products of glycans, i.e. incomplete glycans containing components of the trimannosyl core, but no antennae.

If BP is ignored, the positive CSFs (Figure 6.7) resemble each other as much as the negatives. The A2G0F peaks at 85 min are a similar size and the triplet peaks at 92-95 min have similar patterns. There are, however, large differences in the major peak heights at 80 min (A2G0), 89 min (A2G0FB), 100 min (A2G2F) and 105 min (A2G2FS). The CSF sample RH, for instance, has relatively smaller asialylated agalactosylated peaks at 80 and 85 min (A2G0 and A2G0F), but correspondingly larger digalactosylated and digalactosylated monosialylated peaks at 100 and 105 min respectively (A2G2F and A2G2FS). The sample BP shows a very different pattern of peaks to other samples, with substantial peaks for core products between 60 and 80 minutes. It does not, however, have the increased peaks for sialylated glycans, as seen in the negative sample jt.

The overall pattern of the serum sample RH resembles its paired CSF, but the A2G0F peak at 85 min is larger in the serum, and other peaks between 83 and 92 min differ in the two samples.

c. Data Combined from Both Batches

Overall Peaks

The percentage peak areas of the main peaks in all the CSF samples are shown in Table 6.6, giving the GU value of each peak and the glycan(s) nominated to that GU value. In order to compare the positive samples with the negative controls, the data from this table was plotted as Figure 6.8(A). Also the peak areas of all CSF samples - rather than the percentage areas - were plotted, after a correction, as follows. The areas of the peaks (in mV.ms) were divided by the amount of IgG (in μg) in the dialysates (as given in Table 6.5) and multiplied by the dilution factor for the labelled residues prior to HPLC (Table 6.3). After dividing this value by 10^6 to obtain more manageable numbers, these corrected areas are plotted in Figure 6.8(B): thus in this graph the peaks are relative to the amount of glycan per unit of IgG in the sample. In this graph it appears that the positive samples have more peaks on the left-hand side of the graph, and the negative samples have more on the right-hand. However, this effect is due to the much higher amounts of glycosylation in the positive BP, with large amounts of core products, and in the negative jt, with large amounts of sialylated glycans. If the data for these two samples is removed, the result is seen in Figure 6.9. The graph resembles that of Figure 6.8(A).

It was noted previously that the A2G0F peak height at 85 min appeared similar in the batch 2 negative samples. In fact, in the negative samples in both batches, this peak is always higher than the triplet Gal 1 peaks. In the positive samples, the peak is generally, but not always, smaller than the triplet Gal 1 peaks. Figures 6.8 and 6.9 do not show any difference between the positive and negative samples for this peak, but t-tests were carried out for these peaks as will be shown later.

Figure 6.8(A) shows possibly larger peaks for the positive samples for the three Gal 1 triplet peaks (GU values 6.73, 6.85, and 6.95 in Table 6.6). Also, with the A2G2S

peaks (GU values between 7.98 and 9.23 in Table 6.6), the negative samples seem to predominate. These will be investigated below with graphs and t-tests.

Table 6.6. Percentage areas of the main peaks for all CSF samples.

	Glucose Units	Glycan	GH	DH	AW	HC	AR	RH	BP	ms	dg	ib	jt	al	ja
	4.24								7.0				4.5		
	4.33								8.9				3.4		
	4.43	M3N2	0.5	1.1	0.5	0.5	0.4		0.6	2.0	0.6	2.4	0.1		1.1
	4.62	M3N2F		1.1	0.5	1.2			1.0	0.4	0.8	1.8	0.3	2.5	0.4
	4.68			1.3	0.5	0.6		1.1	1.0	0.3	2.4		0.6		0.4
Core	4.76			0.9				1.1	0.5		0.3		0.2		0.1
products	5.02	M3N3	0.5	3.4	0.2	1.7			7.7	0.7	0.3	0.7	5.1	1.9	1.4
	5.17		0.5	1.2	0.4		0.3	1.6	3.0	0.8		0.4	0.4		1.2
	5.28	M3N3F	0.9	2.5	1.1		1.6		4.0	1.6			0.1		2.0
	5.38		0.3	0.8			0.6	1.1	1.9	0.4			0.3		0.8
	5.50	A2G0	2.2	8.1	4.0	9.6		2.2	1.0	3.3	6.4	3.8	3.5	7.9	4.5
	5.72	A2G0B	1.9	3.4	2.4	0.6	1.7	3.8	5.2	2.3	1.3	1.4		1.3	3.0
	5.83		1.8	3.0	1.3	0.8	5.6		2.4	2.3	0.4	0.7	0.8		2.1
	5.96	A2GOF(α1-6)	12.7	17.4	20.6	23.4	24.4	14.3	7.6	22.6	15.2	18.9	8.1	21.8	25.2
	6.20		1.7	1.5	7.3	9.2	3.6	3.6	3.0	1.8	4.0	6.6	2.3		2.2
	6.29	A2GOFB	6.2	9.4		2.8	2.7	3.1	4.0	5.8	1.7		1.7	5.7	6.6
	6.40	A2G1(α1-6)		2.8			2.7	1.5	1.7	2.2					0.9
	6.47	A2G1(α1-3)	2.2			0.2					0.6	1.1	0.7		1.5
0 sialic	6.60	A2G1(α1-6)B	2.3	2.2	1.6					1.9				0.7	1.9
acids	6.73	A2G1(α1-3)B A2G1(α1-6)F	15.7	10.0	16.8	20.9	19.6	17.4	4.5	12.9	8.4	12.6	5.7	16.4	12.6
	6.85	A2G1(α1-3)F A2G1(α1-6)FB	7.5	3.8	10.8	8.2	9.9	9.2	1.7	7.8	3.6	7.5	3.1	8.9	6.9
	6.95	A2G1(α1-3)FB	7.5	6.0	3.5	4.4	4.9	7.2	5.9	4.1	1.7	2.3	2.1	5.5	4.6
	7.10	A2G2	1.7	1.7	1.2	2.5				1.4	0.7	1.3		0.3	1.6
	7.22		1.2	1.3	1.4			1.5		0.8		1.8	3.5		0.5
	7.35	A2G2B	1.2	1.4					1.9	1.1				0.6	1.0
	7.63	A2G2F A2G2FB	17.6	5.0	15.7	16.0	11.5	16.3	5.0	13.4	19.8	12.0	6.6	15.8	9.4
	7.98	A2G2S1(α1-3)	1.6	1.5	1.4	1.3		1.8	2.1	1.0	2.8	3.7	11.8	1.2	1.1
1 sialic	8.19	A2G2S1(α1-6)	0.1												0.1
acid	8.38	A2G2FS1(α1-3)	7.7	2.5	5.8	7.7	5.9	9.5	4.2	5.9	8.9	7.4	5.2	6.7	3.4
	8.58	A2G2FS1(α1-6)	1.2	1.1		0.5				0.8	1.1	4.4	1.7	1.3	1.2
	8.78	A2G2S2	0.2	0.6		0.2			5.0	0.7	0.5		18.7		0.6
2 sialic	9.14	A2G2FS2	1.1		1.1			1.9	2.1	0.8	2.4	3.2	4.4	1.5	0.6
acids	9.23	A2G2FBS2	1.6	1.0	1.8	1.7				0.8	0.4				1.2

Samples were run on system 1 normal phase HPLC after labelling with 2AB. For method details see section 6.4.7.1. GH to BP are CSF samples containing oligoclonal IgG; ms to ja are control CSF samples without oligoclonal IgG. The GU (glucose unit) values for the peaks are shown in column 2, and the glycans structures comprising the peaks, where identified, are shown in column 3.

A2 indicates biantennary glycans; G (0, 1 or 2) indicates number of galactose residues; S (0, 1 or 2) indicates number of sialic acids; (α1-3) or (α1-6) indicates type of glycosidic linkage; F denotes the presence of fucose, and B denotes a bisecting GlcNAc residue. M and N indicate numbers of mannose or GlcNAc residues respectively in core products.

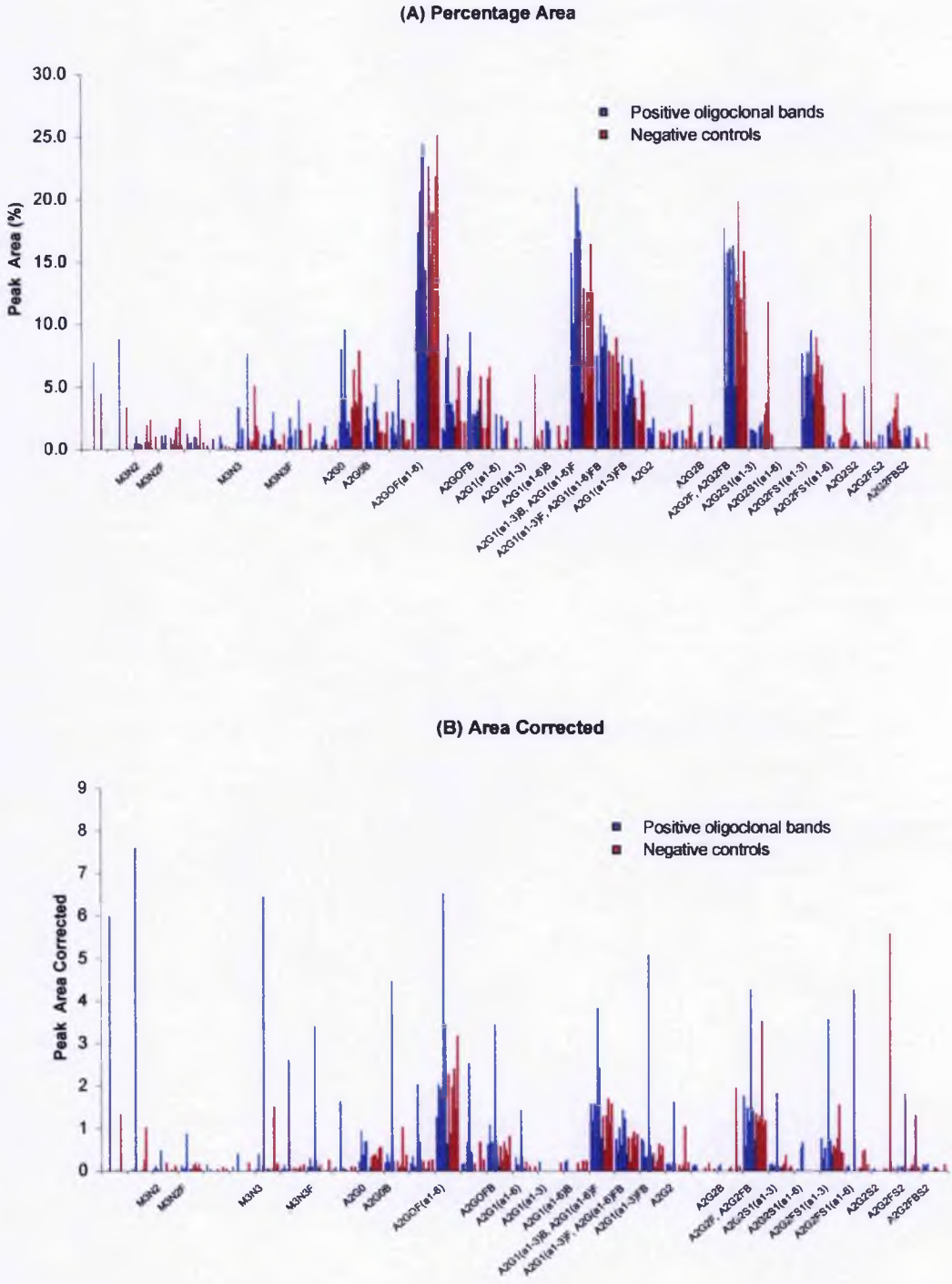


Figure 6.8. Comparison of peak areas of all CSF samples run on system 1 (normal phase) HPLC. For method details see 6.4.7.1. The peaks in (A) are derived from the percentage areas of the HPLC peaks and are drawn from the data in Table 6.6. The peaks in (B) are derived from the absolute areas of the HPLC peaks, with adjustment for the IgG content of the dialysates and dilution of the labelled extract prior to HPLC, as described in 6.5.2.1(c).

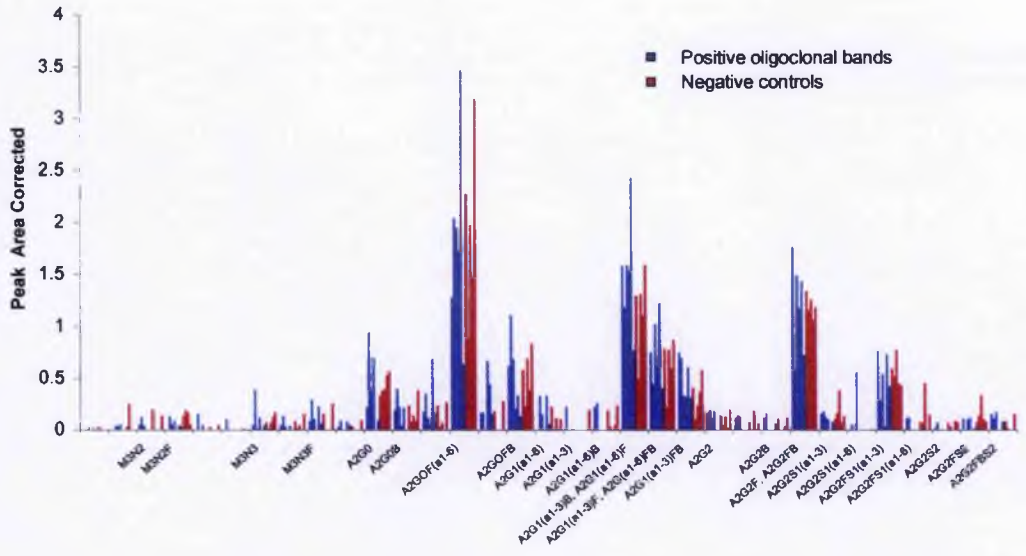


Figure 6.9. Comparison of corrected peak areas for samples on system 1 HPLC: as for Figure 6.8(B) but excluding the samples BP and jt.

Sialylation

To see if sialylation of the glycans could show a difference between the two groups, the peaks were divided into total core products, asialylated, monosialylated or disialylated sections, as indicated by the first column of Table 6.6. For each sample, the peak areas, corrected as described above, in each section were added together and plotted in Figure 6.10. However, the graph does not show any trend in amount or relative proportion of sialylation between the positive and negative groups.

Galactosylation

Peaks were divided into total agalactosylated, monogalactosylated or digalactosylated referring to the data in Table 6.6 (see hatched lines) and plotted in Figure 6.11. However no differences between positives and negatives are revealed.

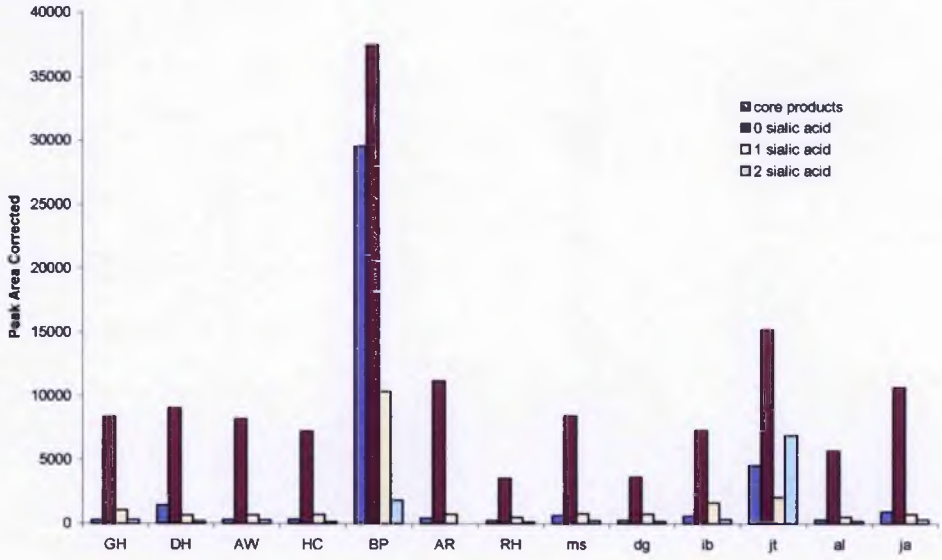


Figure 6.10. Combined corrected areas of peaks for core products, asialylated, monosialylated and disialylated glycans for all CSF samples on system 1 HPLC. For division of peaks into the four sections, please see Table 6.6.

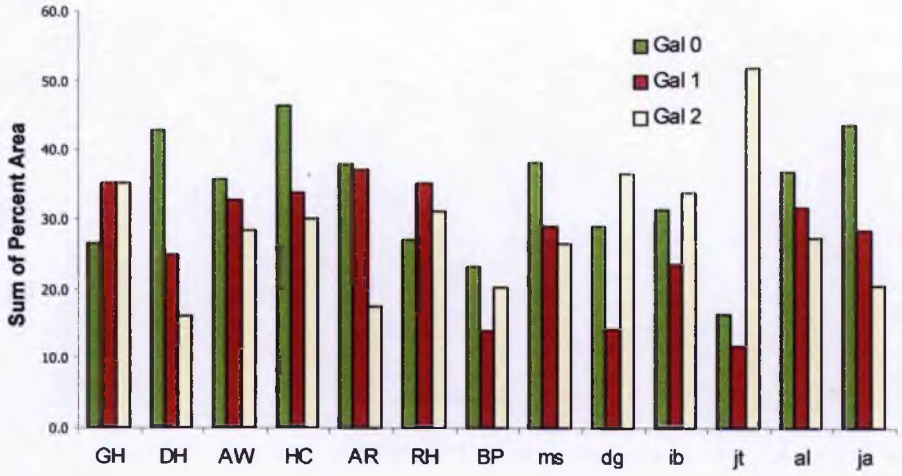


Figure 6.11. Division of peaks for all CSF samples run on system 1 HPLC into total agalactosylated, monogalactosylated or digalactosylated sections (see hatched lines in Table 6.6).

T-Tests

The results of unpaired t-tests, comparing the means of the positive and negative groups of samples, are shown in Table 6.7. Percentage areas of peaks, or the sum of percentage peaks areas were tested, but in some cases, where it was thought appropriate, the absolute areas or the areas corrected for IgG content (described in 6.5.2.1.c) were tested also.

Although the means for the A2G0F peak areas are not different for positives or

Table 6.7. Unpaired t-Tests.

Tested	Positives %Area Mean (±SEM)	Negatives %Area Mean (±SEM)	P values % Area	Absolute Area	Area corrected
A2G0F peak	17.20 (2.30)	18.63 (2.53)	0.6828	0.6779	0.6713
Ratio A2G0F peak/Gal 1	0.57 (0.05)	0.82 (0.06)	0.0078*	0.0141*	0.0145*
Ratio A2G0F peak/Gal 1+Gal 2	0.31 (0.04)	0.34 (0.05)	0.6674	0.7450	
Gal 0	34.21 (3.34)	32.58 (3.87)	0.7542	0.5421	0.4360
Gal 1	30.39 (3.14)	23.02 (3.37)	0.1382	0.4669	0.2647
Gal 2	25.44 (2.82)	32.80 (4.47)	0.1787		
Ratio Gal 0/Gal 1	1.20 (0.15)	1.47 (0.13)	0.2063	0.2152	
Ratio Gal 0/Gal 2	1.49 0.27)	1.15 (0.25)	0.3874		
Ratio Gal 1/Gal 2	1.25 (0.17)	0.82 (0.19)	0.1191	0.1304	0.1609
Ratio Gal 0+Gal 1/Gal 2	2.74 (0.41)	1.97 (0.44)	0.2297		
Ratio Gal 1+Gal 2/Gal 0	0.74 (0.07)	0.60 (0.08)	0.2252	0.6638	
Ratio first/second triplet peaks.	15.00 (2.19)	10.63 (2.00)	0.1752	0.5483	0.2060
S0	79.46 (6.47)	70.85 (7.47)	0.3997	0.6197	0.4762
S1	7.96 (0.92)	11.61 (2.02)	0.1119	0.5190	0.5156
S2	2.62 (0.83)	5.95 (3.44)	0.3330		0.3836
S1+S2	10.59 (1.20)	17.39 (5.11)	0.1902		0.9768
Ratio S1/S0	0.10 (0.01)	0.20 (0.06)	0.1489		0.6088
Ratio S2/S0	0.04 (0.03)	0.13 (0.09)	0.3556		
Ratio S1+S2/S0	0.14 (0.03)	0.33 (0.16)	0.2466		

* Means significantly different ($P<0.05$) between positive (n=7) and negative (n=6) groups.
The P value is calculated on percentage areas of peaks (Table 6.6) and on absolute areas and corrected areas (as described in 6.5.2.1c) where thought appropriate. Throughout n=7 for positive samples; n=6 for negative samples.
The A2G0F peak is at 85 min (5.96 GU); the first and second A2G1 triplet peaks are at 92 and 93 min (6.73 and 6.85 GU) respectively.
Gal 0, Gal 1 and Gal 2 refer to total agalactosylated, monogalactosylated or digalactosylated peaks respectively. S0, S1 and S2 refer to total asialylated, monosialylated or disialylated peaks respectively.

negatives, when the peak areas are expressed as a ratio of the total monogalactosylated peak areas there is a very significant difference. The difference is most pronounced when the percent areas of the peaks is compared (P=0.0078), although is still seen when absolute peak areas (P=0.0141) or corrected areas (P=0.0145) are used. If values for BP and jt are excluded, the difference is still significant (P=0.0138 for percentage area, P=0.0220 for absolute area and P=0.0225 for corrected area). The difference, however, is not revealed when ratios of total agalactosylated (Gal 0) peaks rather than the A2G0F peak are tested, or if the A2G0F peak is compared to combined Gal 1 and Gal 2 peaks.

Low P values, although they are not statistically significant, are also seen for total Gal 1 and total Gal 2 percentage peak areas, and the ratio of total Gal 1 to total Gal 2 percentage peak areas. There are quite large variances with these groups (Figure 6.12), and it is possible that with more data, significant differences may be found.

T-tests did not show significant differences in sialylation, but again, low P values were obtained for total monosialylated peak areas (S1+S2), and the scatter of results

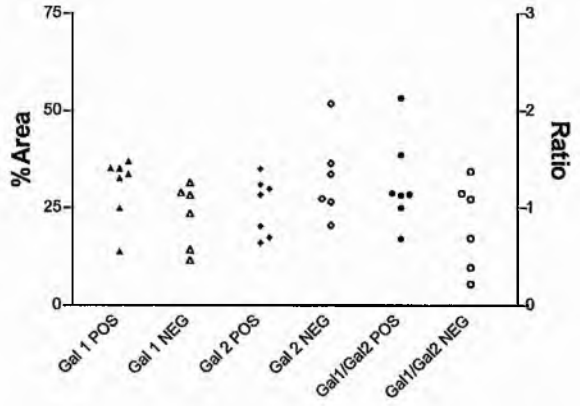


Figure 6.12. Comparison of positive (POS) and negative (NEG) samples for total mono-galactosylated (Gal 1) percentage peak areas, total digalactosylated (Gal 2) percentage peak areas, and the ratio of these - Gal1/Gal2 (on right y axis).

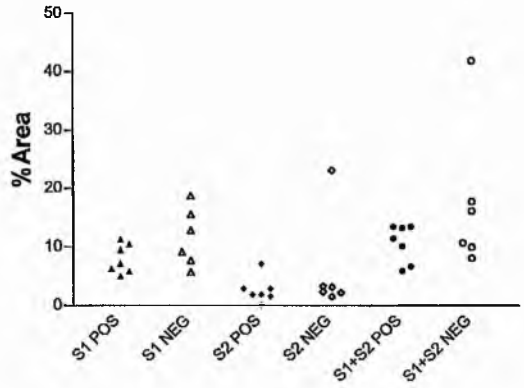


Figure 6.13. Comparison of positive (POS) and negative (NEG) samples for total mono-sialylated (S1) percentage peak areas, total disialylated (S2) percentage peak areas and combined mono- and disialylated (S1+S2) percentage peak areas.

(Figure 6.13) suggests that with more data a difference might be found. Generally, the positive oligoclonal IgG samples show less sialylation than the negative samples.

Total corrected area of all peaks of the positives and negatives were compared, to see if there is a difference in total glycosylation per unit of IgG. The mean total corrected area for the positive samples was 19.28 (SEM=10.02) compared to 12.12 (SEM+3.50) for the negative samples. Although the means are very different, this is due to the extremely high total peak areas for samples BP and jt. If these two samples are excluded for the calculation, the means are similar (9.305±1.14 for positives and 8.81±1.37 for the negatives).

d. Reassessment of Positive and Negative Groups

Because of the difficulty in obtaining CSF samples of large enough quantity for these HPLC studies, the Positive and Negative groups were based on the presence or absence of oligoclonal IgG bands as described in section 6.3. Results of a brain MRI scan or clinical diagnosis of MS (shown in Table 6.1) were not taken into account, as they were

for the lectin assays (see section 4.6.3). If these two criteria are considered, along with the oligoclonal IgG status, the Positive group will consist of three samples - DH, GH and RH - and the Negative group four samples - ib, al, ma and ja. Graphs were plotted as for section 6.5.2.1c, above, for all peaks, sialylated and galactosylated peaks, but no obvious differences between positives and negatives were seen (graphs not shown). Statistical analysis was not possible for this number of samples.

6.5.2.2. System 2. Vydac Protein WAX Column

Chromatograms of the samples of the first batch run on the WAX column are shown in Figure 6.14. (No samples from the second batch were run). Also shown are chromatograms of the water baseline and the fetuin calibrator. The peaks at 2 min contain neutral glycans but also other uncharged material. The broad peak in the CSF samples at 22 min has not been identified. The 1S and 2S peaks are relevant to IgG.

As was found with system 1 (normal phase HPLC) for the first batch of samples, the peak patterns of the negative controls, ja and ms, resemble each other, while the positives show most variability. GH has the largest 1S and 2S peaks of the four samples, while DH has the smallest. This agrees with the quantitation of 1S and 2S peaks for the four samples in system1, as shown in Table 6.6.

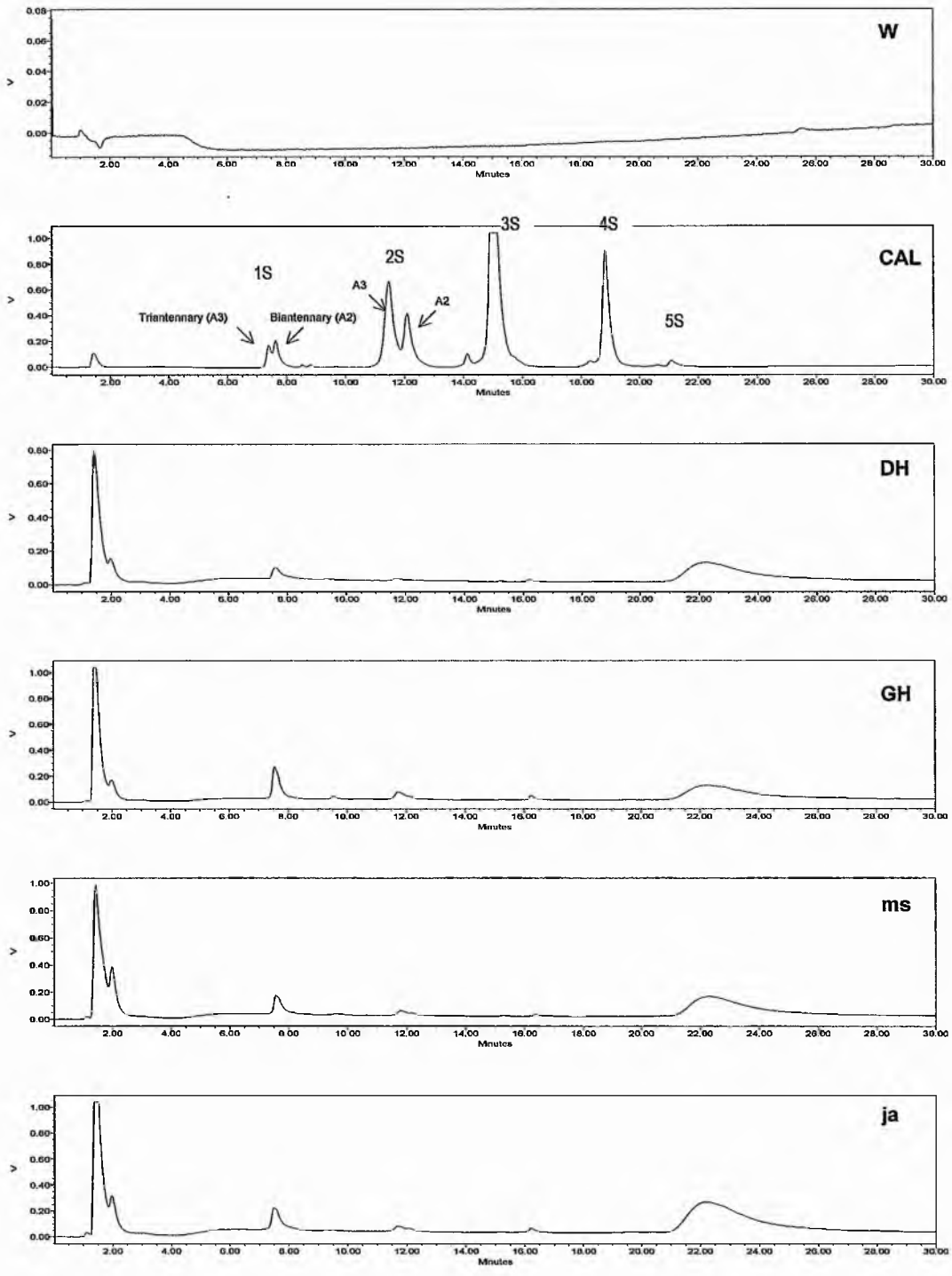


Figure 6.14. Chromatograms of the first batch of samples run by reverse phase HPLC (System 2: WAX column), labelled with 2AB. For running conditions see 6.4.7.2. DH and GH are CSF samples containing oligoclonal IgG bands; ms and ja are CSF samples without oligoclonal IgG. W is water. CAL is the calibrator: bovine fetuin and peak groups are labelled containing monosialylated glycans (1S) and di-, tri-, tetra- and pentasialylated glycans (2S, 3S, 4S and 5S). Also shown is further division into peaks for triantennary (A3) and biantennary (A2) glycans.

6.6. Discussion

The peak patterns for glycans of CSF IgG are generally similar to those found previously for those of serum IgG as shown in Figure 1.10 (Guile et al, 1996). However, a wide variation in patterns was seen with both positive oligoclonal IgG and negative oligoclonal IgG groups of samples. The serum (RH) sample analysed here showed a peak pattern similar to that found previously for IgG in normal serum (Guile at al, 1996), with the corresponding CSF showing moderate differences in some of the peaks. CSF RH compared to serum RH showed a decrease in the A2G0F peak, but increases in the adjacent A2G0FB and A2G0 peaks. Several minor Gal 0 and Gal 1 peaks in the CSF between 86 and 92 minutes are missing in the serum, but there is an increase in the small S2 peaks in the serum between 106 and 000 minutes. It is possible that these differences are due to the oligoclonal IgG in the CSF.

With the wide variation in these samples, consistent differences between the two groups are not easily seen. However, the A2G0F peak is consistently larger in the oligoclonal IgG negative samples, and a significant quantitative difference is found between positives and negatives when the ratio of this peak to the combined mono-galactosyl peaks is tested. In the oligoclonal IgG positive samples the size of the A2G0F peak is similar to that seen in normal serum IgG samples (Guile at al, 1996) and to the serum (RH) sample analysed here.

Previous studies of serum IgG in RA and other autoimmune diseases have found an increase in agalactosylated glycans (Roitt et al, 1988; Tomana et al, 1988; Rook et al, 1989; Furukawa and Kobata, 1991) with a corresponding reduction in mono- and digalactosylated glycans. This is in contrast to the findings here, where the agalatosyl glycans are decreased, albeit moderately, in the samples representing the disease i.e. the oligoclonal IgG positive samples, rather than in the normal controls. Low P values from the Student's t-tests of total monogalactosylated and digalactosylated peaks are not

significant with the small data sets involved here, but do suggest a reduction in monogalactosylation, but not digalactosylation, for the oligoclonal IgG negative samples.

Although significant differences of sialylation were not found for the small numbers of positive and negative oligoclonal IgG samples assayed here, generally the negative samples show an increase in sialylation. Only the batch 1 samples have been run on the system 2 HPLC. The cause of the broad peak at 22 min is not known, but apparently occurs also in all serum samples run on this system. The results generally agreed with the quantitation of the monosialylated and disialylated peaks for the four samples in system 1, with the peak patterns of the negative controls resembling each other, and the positives showing more variability. If the batch 2 samples, with their wide variety of sialylation peaks, were run, differences in sialylation may become clearer.

However, there are several problems with the samples used in this study. One problem is that the normal controls are not from a healthy population, because of the difficulty of obtaining CSF from healthy subjects for ethical reasons. The samples for this project were selected from samples on which routine laboratory CSF analysis had been carried out. Most CSF samples arriving in the laboratory do not contain sufficient IgG for glycan analysis by this HPLC method, which requires a larger amount of IgG than the lectin immunoassays. Even though samples prior to injection onto the HPLC column were diluted between 1 in 4 and 1 in 100, depending on the labelled glycan content, there are many steps during the sample preparation where loss can occur. These include, for instance, loss to the dialysis membrane, and to inside of reaction vessels, evaporation tubes etc. Essentially, the amount of IgG lost at each stage is the same for all samples, regardless of IgG concentration of the sample. So for samples with minimal levels of IgG, the overall loss can be substantial.

The few samples that contain enough IgG do not easily fall into the 'positive MS' or 'negative control' groups that were used for the lectin assays of IgG described in

chapter 4 (see section 4.6.3). For this reason it was decided to use the presence or absence of oligoclonal IgG bands as the investigation criterion, rather than the clinical condition of MS. Therefore, there is the problem (see Table 6.1) that some negative oligoclonal IgG samples may be from patients whose MRI scan suggested demyelination and who show clinical signs of MS. Similarly some oligoclonal IgG positive samples are from patients who have normal MRI scans and for whom MS has not been diagnosed.

An attempt was made to reassess the samples according to the same criteria used for the lectin assays, but with the small number of samples, a difference between the groups was not seen.

As well as selection of samples, there are other factors which affect the results of this study. It would appear that oligoclonal IgG is lacking in A2G0F compared to polyclonal IgG, but because the oligoclonal IgG positive samples as well as the negative controls also contain polyclonal IgG, any subtle differences in glycosylation of oligoclonal IgG would be 'swamped' by the polyclonal glycosylation.

There are several possible reasons for the wide variation of peak patterns, especially of batch 2 samples compared to batch 1 samples. It could be related to the smaller amounts of IgG in the batch 2 samples. Also the resolution of the peaks for batch 2 was poor compared to batch 1, presumably due to the age of the column, and though this is not thought to be a factor in the variation in the peak patterns, it may have affected the accuracy of the quantitation of the peaks.

It is possible that some of the wide variation seen in the batch 2 samples is due to error introduced during the lengthy preparation of the samples, despite the great care that was taken. It is possible that the protein A eluate of one or more samples contained some protein other than IgG, or that during labelling with 2AB there was contamination from other glycans. Two samples especially, the positive BP and the negative jt, have very unusual glycosylation patterns for IgG and are discussed below.

The chromatogram for BP showed excessively large amounts of core products (i.e. trimannosyl structures lacking antennary arms). The usual *N*-glycan pattern appears to be present, although it is not clear. The large scale of the y-axis of the chromatogram shows that the glycan structures are generally in much larger quantities than in the other samples. The clinical diagnosis is 'possible 1° progressive MS', although an MRI brain scan has never been performed. It is not known why there should be so many non-antennary glycans, unless these occur with deterioration of the sample from the action of glycosidases. Contamination of the sample should be suspected.

The sample jt contains very large amounts of mono- and disialylated glycans, the peaks of which dominate the chromatogram. The usual amounts of *N*-glycan peaks are still there, although they are dwarfed on the chromatogram. It also contains a relatively large amount of core products. There are no clues as to the cause of the high sialylation from the clinical details. The patient showed 'upper motor neurone signs', not thought to be MS, but questionably 'familial spastic paraplegia'. The MRI brain scan was normal. Again contamination of the sample should be suspected, although this could be ruled out if sialic acid could be shown to be increased in the original CSF sample.

Chapter Seven

Discussion

7.1. The Problem Investigated

Multiple sclerosis (MS) is an autoimmune disease, characterised by the breakdown of myelin in the brain (Adams, 1989). The disease mechanism is poorly understood and the causative agent and the actual autoimmune target are unknown (Bannister, 1992; Zanetta, 1996).

IgG is involved in the disease, not simply as an antibody, in common with other autoimmune diseases (Roitt et al, 1998), but also because oligoclonal IgG is produced by lymphocytes within the CNS (Adams, 1989). The presence of oligoclonal IgG in CSF is often used as an aid to diagnose MS, but it is not specific to MS, nor does it always occur in the disease, and its prevalence is not related to the severity of the disease (Fukuzawa et al, 1998). It has not been shown conclusively that oligoclonal IgG an autoantibody directed against myelin (Adams, 1989; Rand et al, 1998), but it may possibly assist in the elimination of antigenic fragments of proteins, such a myelin basic protein (MPB), broken down from myelin by proteases triggered by cytokines (Opdenakker and Damme, 1994).

A feature of oligoclonal IgG is that a large proportion has a high isoelectric point (pI) compared to polyclonal IgG, as seen with isoelectric focusing (IEF) (Cowdrey et al, 1990). One of the aims of the project has been to see if this high pI is due to variant glycosylation of the IgG, such as a deficit of sialic acid. Other causes could be an increase of basic amino acids in the IgG, or the complexing of IgG with fragments of basic peptides, for instance from the breakdown products of myelin (Opdenakker and Damme, 1994).

The glycosylation of IgG and other proteins and conjugates plays a key role in immunity and likewise in autoimmune disease (Gabius et al, 1997; Rudd et al, 1999). A number of inflammatory diseases are associated with a deficiency of terminal galactose on the oligosaccharides of the F_c fraction of IgG in serum (i.e. Gal 0 or agalactosyl IgG). These include rheumatoid arthritis (RA) and osteoarthritis (Parekh et al, 1985; Roitt et al, 1988), systemic lupus erythematosus, Crohn's disease and tuberculosis (Tomana et al, 1988; Furukawa and Kobata, 1991). In other diseases, a variety of changes in glycosylation has been found. Cancer cells, for instance, often have increased sialylation and glycan branching on their surface, and some types also show differences in galactosylation and fucosylation (Brockhausen, 1993). Also in cancer there may be an expression of carbohydrate antigens, usually *O*-linked glycans, that are not normally present in the particular tissue (Kim et al, 1996). The fucosylation of serum alpha-fetoprotein shows differences in liver germ cell tumours compared to hepatomas (Aoyagi et al, 1998). Human chorionic gonadotropin (HCG) in urine from women with invasive hydatidiform mole has been found with additional triantennary chains compared with urine from women in normal pregnancy, and HCG with abnormal biantennary chains has been found in urine from patients with choriocarcinoma or non-invasive mole (Kobata and Takeuchi, 1999). Asialylation of serum transferrin is found in liver disease (Matsumoto et al, 1994), alcoholism (Stibler et al, 1980) and in inherited metabolic diseases (Keir et al, 1999).

It is possible, therefore, that the glycosylation of IgG might be different in MS patients compared to normal controls. In this project the IgG in CSF has been studied and as mentioned already, two types of IgG can exist in CSF. There is polyclonal IgG, which is produced by the pool of lymphocytes circulating in the body outside of the CNS. Levels of this may increase in response to disease such as infection, and may also increase in the CSF. Additionally, there is oligoclonal IgG produced within the CNS by a relatively small numbers of CSF lymphocytes. This is found in CSF only in certain neurological

diseases. The glycosylation of each of these types of IgG may be under different control mechanisms. For instance, with an inflammatory disease such as MS, serum polyclonal IgG, and hence CSF polyclonal IgG, might have an abnormal feature in the same way that serum IgG in RA is less galactosylated. However, the glycosylation of the oligoclonal IgG might be independent of this. Therefore changes in glycosylation of the polyclonal IgG might be hidden by the oligoclonal IgG. The reverse might also be true: oligoclonal IgG might be glycosylated differently from polyclonal IgG (e.g. asialylated), but detection of this may be obscured or confused by the presence of polyclonal IgG. In this project, the lectin binding assays and the HPLC analysis do not distinguish between the two types of IgG. IEF, however, can differentiate between the bands of oligoclonal IgG and the diffuse pattern of polyclonal IgG.

7.2. The Findings

In this section, the findings for each of the methods (Chapters 3 to 6) will be looked at in turn.

7.2.1. Chapter 3: Development of an IgG Immunoassay

Essentially this is a methodological chapter, and does not deal with the problems outlined in 7.1, above. Setting up a method to quantitate CSF IgG was a necessary step for the development of the lectin binding assays. It resulted in a robust, highly sensitive method for IgG with reasonable precision and good recovery of IgG. A good correlation ($r=0.88$, $n=68$) was found with an established immunoturbidometric assay for CSF IgG. Although attempts were made to automate the assay, they were not successful. The outcome was a reliable assay in which CSF IgG was 'captured' in a quantitative way, which facilitated the next step of incorporating lectins into the assay in order to probe the IgG carbohydrates.

7.2.2. Chapter 4: Development of Lectin Binding Assays for CSF IgG

Much of this chapter was concerned with method development and overcoming problems with interfering substances in the CSF samples. Interference was thought to be from highly glycosylated substances in the CSF, which bound non-specifically to the capture antibody as well as to the assay tube, and were then bound by the lectins. These were finally eliminated by using protein A affinity chromatography to isolate the sample IgG. Again attempts to automate the assays were unsuccessful.

Four different lectins - Con A, RCA, SNA and DSL - were used to look at the carbohydrates in IgG in two groups of CSF samples. The MS positive group consisted of 13 CSF samples from patients diagnosed as having MS, containing oligoclonal IgG and raised [IgG]/[total protein] ratio. The Control group consisted of 14 CSF samples, from patients without the diagnosis of MS, containing no oligoclonal IgG and normal [IgG]/[total protein] ratio.

A significant difference between MS samples and controls was found for Con A binding to IgG (unpaired t-test: $P= 0.0172$; $n=13$ and 14). The mean for the MS samples was higher, with a wider spread of results. The amount of IgG added in the immuno-assays was the same for all samples. Since Con A primarily binds to mannose, these results suggest that the IgG in the MS samples contains more mannose. This could mean either that the extent of IgG glycosylation in the MS samples is increased, i.e. that there are more glycans per IgG molecule, or that the extent of glycosylation is the same but that the glycans contain more mannose, i.e. have more branching. Serum IgG glycans are always described as having a 'trimannosyl core' (see sections 1.2.5.1 and 1.3.4.2) with up to 2 antennary chains. It is unlikely that CSF IgG is fundamentally different, so an increase in average number of glycans per IgG molecule is more likely.

However, another possible reason for increased Con A binding is that the more terminal monosaccharides, i.e. sialic acid and galactose, might be missing from the glycans

in the MS samples, causing the mannose residues to be more exposed and more accessible to Con A binding. Glycans lacking antennary chains altogether, exposing the mannose, often called 'core products', were detected by HPLC of CSF samples in this project but overall differences between the oligoclonal IgG positive and negative groups were not seen. However, as will be made clearer in the discussion of the HPLC results, although galactosylation might be increased, sialic acid is possibly reduced in oligoclonal IgG so this could be a reason for increased Con A binding rather than an increased number of glycans per IgG molecule.

Con A binding to IgG was also found to correlate with the %IgG values of the samples analysed (Paired t-test: $P=0.0443$; $n= 27$). This is to be expected if Con A does distinguish between MS and non-MS conditions, because %IgG is known to correlate with MS and is used clinically as one of the markers for the disease (see section 1.4.9). There may be the potential for Con A binding to IgG to be used as a disease marker in place of, or in addition to, %IgG. For the samples analysed in the project, %IgG and IgG concentration gave better differentiation between MS positives and controls than Con A binding (Unpaired t-tests: $P<0.0001$; $n=13$ and 14 for both %IgG and IgG concentration; $P=0.172$; $n=13$ and 14 for Con A). But this is not surprising, since increased %IgG and IgG concentration were part of the criteria used for selecting MS positive samples for the study. So the potential of using Con A binding as a marker could be explored by analysing new samples selected on the basis of diagnosis of MS and other criteria apart from IgG levels.

RCA binding to galactose was substantially increased in 3 out of the 13 MS samples compared with the controls, but the difference between the two means was not quite significant at the 95% confidence level ($P=0.0981$). There appears, therefore, to be increased galactosylation in only some of the MS positive samples. A correlation was not

found between Con A and RCA (r=0.0650; P=0.7474), although the three high RCA-binding samples also had high Con A binding.

For SNA, no statistical difference was found between the MS samples and controls (Unpaired t-tests: P= 0.4506, n= 13 and 13). However, the scatter of binding to the MS samples was much wider than the controls, with 5 out of the 13 MS results being higher than any of the controls. Since SNA binds to sialic acid, the increased binding of SNA in a large proportion of the MS samples does not appear to support the theory that oligoclonal IgG is deficient in sialic acid. This will be further discussed later.

There was, however, a strong correlation between SNA and RCA binding (r=0.7924; P<0.0001; n=26). This is consistent with the understanding that where galactose is deficient, sialic acid must also be lacking.

DSL binding did not distinguish at all between MS samples and controls (Unpaired t-test: P=0.6922; n=13 and 14), with similar means and wide variances, but also small RLU counts compared to the other lectin assays.

As discussed in section 4.7, one of the problems with using lectins to investigate the glycans of a protein is the possibility of steric hindrance to their binding by the protein. This can be overcome by denaturation of the protein, but for this project it was chosen not to do this, to enable isolation of the IgG from the samples by immunoassay. It is suspected that the relatively low binding generally in the DSL assay is due to steric hindrance. The moieties which are bound by DSL - $GlcNAc(\beta1\text{-}4)GlcNAc]_{1\text{-}3}$ and $Gal(\beta1\text{-}4)GlcNAc$ - are present in all glycan chains of IgG (found to date) so DSL binding would be expected to be as extensive as Con A binding to mannose. Although binding of DSL to IgG appeared to be high in the preliminary lectin assays, this binding was probably predominantly due to the interferent. A lectin's binding to its target carbohydrate in a glycoprotein could be influenced to varying degrees by neighbouring

groups which may be variable or absent, so binding to the target may not be as quantitative as one would hope.

Overall, the lectin assays have shown that there is a difference in IgG glycosylation in the MS positive samples compared to controls, with generally a higher mean and greater spread of results in the MS samples. This might be due to variant glycosylation of oligoclonal IgG compared to polyclonal IgG or to changes in the glycosylation of the polyclonal IgG with MS, or a combination of both. These points will be looked at further later.

7.2.3. Chapter 5: Isoelectric Focusing of CSF and Detection of Glycosylation using Lectins

The work using IEF to investigate the glycosylation of IgG is of a preliminary nature and could still be developed further. Use of pH 3 to10 gel strips and detection with lectins was found to be difficult, however the high resolution of the bands on some of the PVDF blots illustrates the potential of the technique.

Con A was seen to bind oligoclonal IgG bands throughout the pH 3 to 10 range, showing that mannose-containing glycans are present throughout the pI range of oligoclonal IgG. SNA, however, although only a few samples were run, was seen to bind oligoclonal IgG in the acidic and neutral regions only, and not in the alkaline region. This suggests that alkaline oligoclonal IgG is possibly deficient in sialic acid.

This finding with SNA, however, does not agree with the findings of the lectin binding assays, where SNA binding was certainly not reduced in the MS samples, and in a few MS samples it was higher than all the controls. If the validity of both results is to be believed, it could be argued that the alkaline oligoclonal IgG is indeed deficient in sialic acid, but neutral or acidic oligoclonal IgG might have increased amounts of sialic acid. Another explanation is that there may be increased sialic acid in the polyclonal IgG in MS

samples compared to controls, which may overcome the effect of the sialylation of the oligoclonal IgG. These points will be discussed further later.

Another aspect of using IEF was to study the effect of deglycosylation of oligoclonal IgG. A CSF sample containing oligoclonal IgG bands was subjected to IEF before and after treatment with PNGase F. Confirmation of deglycosylation of the treated sample was made by its absence of binding to Con A. But detection using the anti-IgG double antibody system showed a pattern of banding that was different to the untreated sample, although it was as extensive. This was interpreted as suggesting that the spread of isoelectric points of oligoclonal IgG bands is not simply due to differences in carbohydrate. Were this the case, the oligoclonal IgG bands would coalesce into a single, or only a few, bands.

7.2.4. Chapter 6: Analysis of IgG Glycans by HPLC

The selection of samples for HPLC analysis was different from the lectin immunoassays, and this has important implications when comparing the results from the two methods. Because of the difficulty of finding CSF samples with sufficient IgG for the HPLC method, the more stringent selection criteria of the lectin immunoassays was not used. Rather than selecting on the basis of the clinical condition of MS (accompanied by positive oligoclonal IgG bands in the CSF, raised CSF IgG and an MRI scan indicating demyelination), the positive samples for HPLC were simply selected on the basis of containing oligoclonal IgG. Similarly the negative 'controls' were selected as not containing oligoclonal IgG.

As explained in section 7.1, neither the HPLC method nor the lectin immunoassays can distinguish between oligoclonal and polyclonal IgG. Therefore differences in glycosylation between the positive and negative groups may be due to one, or both, kinds of IgG. A difference found by the lectin immunoassays can be attributed to MS, although it may be due to a characteristic of the oligoclonal IgG, or to the

polyclonal IgG, or both. However, a difference found by the HPLC method probably cannot be related to disease and again may be a reflection of oligoclonal or polyclonal IgG, or both.

The HPLC samples were analysed in two batches. In the first batch, the two negative samples showed similar peak patterns while the positive samples showed wider variations. In the second batch there was more variation in the peak patterns for the negative as well as the positive sample groups. Several possible reasons for this increased variation have been given for this in the Chapter Six discussion (section 6.6). It is difficult to tell, especially with few previously published examples of HPLC of IgG glycans, whether the variation is due to post-transcriptional heterogeneity, or whether inaccuracy has been introduced into the assay.

Two samples especially, BP and jt, show extremes of variation. The variation in jt cannot be due to oligoclonal IgG because it contains none. Omission of the two samples from statistical comparison of positive and negative groups does not greatly affect the results. It is possible that these two samples have been contaminated because of their atypical peak patterns and because they contain a much larger quantity of glycan structures than the other samples. However, the peak patterns of these two samples do not have the characteristics of contamination that have been seen at the Glycobiology Institute. It is possible that they are genuine results, and that the patients are suffering from different inherited metabolic disorders of glycosylation. The result for BP, especially, with an excess of trimannosyl core products with some GlcNAc residues, suggests a defect in a GlcNAc transferase or perhaps a galactosyltransferase. The case notes of these two patients will be re-examined for any evidence of symptoms of inherited disease.

The main finding of the HPLC analysis is a difference in the A2G0F peak, which is consistently larger in the negative samples compared to the positives. The difference is statistically significant when the A2G0F percentage peak area is expressed as a ratio of the

total monogalactosylated (Gal 1) percentage peak area. In previous studies, increased levels of agalactosylated glycans of serum IgG have been found in patients with RA and other autoimmune diseases (Roitt et al, 1998; Tomana et al, 1988; Rook et al, 1989; Furukawa and Kobata, 1991). This appears to be in contrast to the case here, where the agalactosyl glycans are decreased in the samples representing the disease, i.e. the oligoclonal IgG positive samples. However, as previously explained, positive and negative sample groups for the HPLC analysis were not selected on the basis of MS status, i.e. the oligoclonal IgG positive samples are not necessarily MS positive samples. Therefore the decrease in A2G0F glycans in the positive samples might well stem from the oligoclonal IgG and might not be due to polyclonal IgG. There might be separate changes in the polyclonal IgG.

In comparisons of galactosylation, the Student's t-test was also applied to total monogalactosylated (Gal 1) and total digalactosylated (Gal 2) percentage peak areas and the ratio of Gal 1 to Gal 2 percentage peak areas for the two sample groups. Although not statistically significant, low P values were found and scatter plots suggest that the oligoclonal IgG positive samples contain more Gal 1 glycans but less Gal 2, but overall are more galactosylated than the negative oligoclonal IgG samples.

As well as percentage peak areas, t-tests were applied to the absolute peak areas, and the absolute peak areas corrected according to the amount of IgG in the dialysates and the dilution of the labelled extract prior to column injection. The correction would adjust the area to relate to the amount of glycan per unit of IgG in the CSF sample. Although differences between the two sample groups, using these values, were significant in some cases, the P values were never lower than the corresponding ones for percentage areas.

Total glycosylation, i.e. the sum of all peaks, was also looked at in terms of absolute peak areas and corrected peak areas, but no difference was found between the positive and negative groups.

Differences in sialylation between the positive and negative sample groups were not significantly different, however low P values were obtained, with small differences in the means but a wide scatter of results. With more data a significant difference might be found. Generally, the positive oligoclonal IgG samples were less sialylated than the negatives.

A single serum sample was analysed along with its paired CSF. Generally the peak patterns of the two were similar, but some differences are notable. We see in the CSF a decrease in the A2G0F peak, although there are small increases in minor G0 and G1 peaks. We also see a decrease in disialylated peaks in the CSF sample, while the monosialylated are similar. The differences might be due to the influence of oligoclonal IgG which is present in the CSF and not in the serum, while the polyclonal IgG in both samples is from the same pool. The two differences - a decrease in A2G0F glycans and decreased sialylation - correspond to the differences found for oligoclonal positive CSF samples compared to the negatives. It could be, therefore, that these are both features of oligoclonal IgG rather than polyclonal IgG, although these tentative conclusions are based on only one sample.

There is another point to consider with the HPLC results. One possible reason suggested for the alkaline nature of the oligoclonal IgG, as well as a possible deficiency of sialic acid, is that IgG might be complexing with degradation products of myelin, such as myelin basic protein (MBP), resulting from protease activity (Opdenakker and Van Damme, 1994). Although MBP itself is not glycosylated, other myelin components are, such as myelin-associated glycoprotein (MOG) which contains 8 or 9 *N*-glycosylation sites. If these IgG-degradation product complexes bind tightly to oligoclonal IgG, these

complexes would have been bound by protein A during affinity purification of the CSF samples for HPLC. Elution of IgG with pH 2.8 phosphate-citrate buffer would release the peptides into the eluate with the IgG. As the molecular weight cut-off of the dialysis membranes subsequently used was 3500 kDa, the peptides would probably not be removed from the dialysates, and any glycans attached would go on to be labelled with 2AB, along with the IgG glycans, and hence contribute to the HPLC chromatograms. It is not certain if this has occurred, or if so, how much the peptide glycans contribute to the HPLC chromatograms. It might explain the diverse peak patterns obtained for the CSF samples, although the oligoclonal IgG negative samples should not be affected, and it does not account for the unusual peak pattern for sample jt, which is oligoclonal IgG negative.

7.3. Have the Questions Been Answered?

The aims of the project were set out in Chapter One (section 1.5). In this section the combined findings of the three methods of glycosylation analysis will be discussed in relation to the questions asked concerning MS. The interpretation of the results is complicated by the differences in the selection of positive and negative samples for the lectin immunoassays and HPLC, but also by the occurrence of both polyclonal and oligoclonal IgG in CSF each of which may possibly show different patterns of glycosylation.

7.3.1. Changes in IgG Glycosylation

Does glycosylation of CSF IgG change in MS compared with normal? The results of the lectin immunoassays, which looked at CSF from MS patients and non-MS patients, indicate that there is probably a difference in IgG glycosylation. *If there are changes, what are they in terms of the glycans or the monosaccharides of IgG? Is the glycosylation pattern of IgG in CSF the*

same as that in serum? These points will be discussed by looking in turn at each general glycosylation change, in the light of results of the three analytical methods.

7.3.1.1. Extent of Glycosylation

A possible difference between normal IgG, and IgG from patients with a disease, could be the average number of glycans per IgG molecule. A significant increase in Con A binding to IgG was found in the MS positive samples compared to the negative controls. This could be due to increased glycosylation of the IgG, although there are other possible reasons. The IgG glycans could contain more branched mannose, although this would be very unusual, with hitherto only trimannosyl glycans being recorded in IgG. Or the increased Con A binding could be due to a possible lack of sialic acid in the oligoclonal IgG, giving Con A more access to the mannose.

The suggestion of increased glycosylation of IgG glycans in the MS positive samples is supported by the increase in RCA binding to galactose. However, total absolute areas of HPLC peaks, and the absolute areas corrected according to the IgG in the dialysate and dilution of the labelled extract, were calculated and differences between the oligoclonal IgG positive and negative samples were examined. No differences were seen, although they could be masked because the samples after dialysis contained varying amounts of IgG and variable losses of IgG could occur during subsequent sample preparation.

In short, the increased Con A binding in the MS positive samples is not supported by the HPLC data. It is possible, though, that this is because a different category of positive samples was analysed by HPLC. IEF gives no information about extent of glycosylation of the IgG molecule: although Con A binding to IgG bands in samples containing oligoclonal IgG was greater than to the IgG bands in oligoclonal IgG negative samples, this was not looked at quantitatively.

7.3.1.2. Galactosylation

The results of the RCA immunoassay suggest an increase in the galactosylation of the MS positive samples compared to the negative controls. With HPLC, a decrease in the A2G0F/Gal 1 ratio was noted in the oligoclonal IgG positive samples, suggesting a tendency toward monogalactosylation rather than agalactosylation although di-galactosylation was possibly greater in the negative samples. The results of the two methods appear to agree with each other. The increase in galactosylation of the positive groups of samples could be from either the oligoclonal or the polyclonal IgG in the samples. Galactosylation was not studied with IEF.

A serum sample, with a paired oligoclonal IgG positive CSF was also assayed by HPLC. In the serum, the A2G0F peak area was increased compared to that in the CSF, and is thus more like the A2G0F peak in the oligoclonal IgG negative CSF samples. Assuming that the IgG in serum is polyclonal rather than oligoclonal, this would suggest that the increased A2G0F peak is due to polyclonal, rather than oligoclonal, IgG, i.e. there are less A2G0F glycans in oligoclonal IgG. It would explain why decreased agalactosyl IgG was found in the oligoclonal IgG positive samples in this study, when increased agalactosyl IgG is a feature of several autoimmune diseases. It is possible that separate changes, such as an increase in agalactosyl IgG, occur in the polyclonal IgG of CSF and serum in MS, but in CSF are confused by oligoclonal IgG glycosylation.

7.3.1.3. Sialylation

SNA binding to IgG in MS positive samples showed a wider and higher spread of results compared to the controls. Although the means were not significantly different (unpaired t-test: P=0.4506; n=13 and 14), 5 out of the 13 MS results were higher than the controls, with 2 results lower.

With HPLC, the data suggests that the oligoclonal IgG positive samples were less sialylated than the negative samples, although again significant differences between

oligoclonal IgG positive and negative samples were not seen (unpaired t-test for S1+S2 percentage peak areas: P=0.1902; n=6 and 7). This is contrary to the lectin immunoassay results. The HPLC chromatogram for the serum sample shows increased peaks of sialylated glycans compared to that of the paired CSF, also suggesting that sialylated glycans are increased in polyclonal IgG compared to oligoclonal IgG.

SNA detection of bands was used for only a few samples run by IEF. However, the lectin appeared not to bind the oligoclonal IgG in the alkaline region, although it bound IgG in the neutral and acidic regions. This suggests that oligoclonal IgG contains less sialic acid than polyclonal IgG. This agrees with the HPLC finding.

The contradictory results of the SNA immunoassay are difficult to explain. Although the MS positive samples exhibit a wide range of sialylation, the picture is certainly not of reduced sialylation. The MS positive samples selected for the lectin immunoassays differ from the oligoclonal positive samples selected for HPLC in that they are all MS cases and perhaps the anomaly is related to this. There might be a tendency for increased levels of polyclonal IgG to occur in this sample group along with the oligoclonal IgG, and the increased sialylation to result from this. On the other hand, increased polyclonal IgG may not be a feature of all the samples in the oligoclonal IgG positive group selected for HPLC, so increased sialylation is not seen.

7.3.2. Oligoclonal IgG

What is the character of the oligoclonal IgG bands? The IEF studies suggest that, while variable glycosylation occurs in the oligoclonal IgG bands, the spread of isoelectric points of the bands is not simply due to differences in carbohydrate. The lectin immunoassays and the HPLC assays do not distinguish between oligoclonal and polyclonal IgG, but a clue to the features of oligoclonal IgG might be given by comparison of the serum sample with its paired CSF sample, which contains oligoclonal IgG. It is possible that differences between the two might be due to the difference between oligoclonal IgG and polyclonal

IgG. However, assumptions based on the results of a single serum/CSF pair are not definitive.

Looking at the results from all three methods, it is possible that oligoclonal IgG glycans are generally less sialylated than polyclonal IgG glycans, although this inference is not backed up by the lectin immunoassay data. The results also suggest that oligoclonal IgG glycans are generally more galactosylated. There is a slight anomaly here, for a lack of galactose would perhaps necessitate a lack of sialic acid, which is the final residue on IgG chains, but the converse does not necessarily apply.

The impression gained from considering all results is that there is a wide variation of glycosylation in oligoclonal IgG compared to polyclonal, but that the glycans are generally more galactosylated and less sialylated. The question of whether a deficit of sialic acid is responsible for the alkaline nature of the oligoclonal IgG is still not completely answered. It seems that sialic acid is reduced in oligoclonal IgG, but is not the primary cause of its alkalinity. If it is caused by the complexing of IgG with alkaline peptides, such as fragments of MBP, fragments of other myelin components might also be forming complexes and possibly contributing glycans to the patterns of the HPLC chromatograms, which would negate many of the conclusions drawn in this project. Another reason suggested for the alkaline nature of IgG was a change in the amino acids of the IgG. Could the amino acids in the variable region of the Fab fraction of the oligoclonal IgG be different from that of polyclonal IgG in that either they are more basic, or they have a predilection for glycans which are deficient in sialic acid? IgG research in the past has concentrated on the glycans in the Fc fraction, for instance the finding of agalactosyl IgG in the serum of RA patients is due to differences in Fc glycans (Parekh et al, 1985). Perhaps Fab glycans could also show changes in different diseases.

7.3.3. The Effect of the Glycosylation Changes

Do the changes (if any) contribute to the pathogenesis of MS and, if so, how? Specific differences in the glycosylation of polyclonal IgG between MS samples and controls have not been established in this project, but it has been found that oligoclonal IgG compared to polyclonal may be less sialylated, and possibly more galactosylated.

One of the functions of sialic acid in glycoconjugates is to act as a shield to prevent recognition by clearing receptors (Powell and Varki, 1995). An important receptor is the IgG Fc receptor of macrophages which will remove from circulation glycoconjugates devoid of sialic acid (Paulson, 1989; Wormald et al, 1997). This supports the theory that oligoclonal IgG could help in the elimination of specific antigens such as peptide fragments from myelin (Opdenakker and Damme, 1994). Galactose is also important for binding of IgG to C1q and Fc receptors (Tsuchiya et al, 1989).

IgG contains on average 2.4 glycans, so with two conserved glycans in the Fc region, about one in two molecules also has a glycan chain in the hypervariable region of the Fab. The Fab glycans contain higher levels of galactose than the Fc glycans (Wormald et al, 1997). The possible increase in galactose in the oligoclonal IgG could be in either the Fc or Fab glycans. An increase in overall glycosylation for the MS positive samples was perhaps suggested by the results and could perhaps be due to increased Fab glycans in the oligoclonal IgG compared to IgG.

Without speculating, it is not possible to elucidate any further the biochemical or clinical significance of the glycosylation changes found in this project.

7.4. Future Work

One of the points to come out of this research is the need to look at polyclonal IgG separately from oligoclonal IgG when investigating possible glycosylation changes in neurological diseases. Therefore two continuing lines of research are suggested: firstly, the

study of possible glycosylation changes in polyclonal IgG in neurological diseases such as MS and, secondly, further investigation of the character of oligoclonal IgG and its role in neurological diseases such as MS.

Changes in polyclonal IgG glycosylation have been described for several autoimmune diseases, with serum IgG as the sample (Tomana et al, 1988; Rook et al, 1989; Furukawa and Kobata, 1991; Rudd and Dwek, 1997; Roitt et al, 1998). In this project, using CSF as the sample has made interpretation of the data difficult because of the probable different glycosylation of oligoclonal IgG, and better results might be obtained with future studies of neurological diseases by analysing serum IgG samples, rather than CSF, when comparing patients with controls.

The glycosylation pattern of the polyclonal IgG in CSF probably reflects the glycosylation pattern of IgG in the serum, unless it undergoes glycosylation changes during its passage across the blood-brain barrier. This could be demonstrated by analysing paired samples of serum and CSF from 'normal' control subjects, where IgG in both would be polyclonal. If the paired samples from normal subjects have matching glycosylation patterns, then analysis of paired samples from MS patients, or from patients with other neurological diseases where CSF oligoclonal IgG is a feature, might show a difference, which would be due to the oligoclonal IgG. Subtraction software has been developed at the Oxford Glycobiology Institute, which could reveal the glycosylation profile of oligoclonal IgG. Comparison of oligoclonal IgG in neurological diseases as well as MS might show glycosylation features which could lead to a more specific diagnostic test for MS.

Various studies have been carried out to identify the antigenic target of oligoclonal IgG. Activity against different viruses has been found using phage libraries (Cortese et al, 1996; Rand et al 1998) and against different components of myelin, including myelin basic protein (MBP), using IEF with immunoblotting techniques (Cruz et al 1987). But no

single antigen common to more than 50% of the MS patients, has been found in any of the studies. Another approach would be to identify the peptide fragments that might be bound to the IgG. One technique that would enable analysis of peptide fragments in the presence of a large molecule like IgG is mass spectrometry, such as MALDI, which has high sensitivity over a wide range of molecular weights. The fragment peptides should be distinguishable from the IgG, and their mass could also be determined. SDS PAGE might be another technique for looking at oligoclonal IgG complexes: under SDS conditions, any peptides complexed with IgG would be disassociated, and would migrate according to their mass, which could be determined by using calibrators. A problem with this technique might be lack of sensitivity and the difficulty of detecting a small amount of peptide in the presence of a larger amount of IgG. But visualisation of the bands blotted from the gel might be possible with a sensitive stain such as colloidal gold or by using monoclonal antibodies specific, for example, for different regions of MBP. Use of two-dimensional electrophoresis might also give added sensitivity to IEF or SDS-PAGE techniques.

Although oligoclonal IgG may not be the cause of myelin breakdown, its study will hopefully lead to a better understanding of MS, and might possibly lead to better treatment. Detection of oligoclonal IgG in the CSF is currently an important diagnostic tool, but further study might also lead to a more specific marker for MS.

Appendix

Table of GU Values

The following table shows GU values for *N*-linked glycans for the HPLC System 1 used for this project, taken from tables being prepared by the Oxford Glycobiology Institute. GU values were derived at the Institute by comparing the retention times of peaks of 2AB labelled standard glycans with a series of glucose homopolymers (dextran ladder), derived by partial hydrolysis of dextran, that was run simultaneously. Subsequently, incremental GU values were calculated for additions of monosaccharides to the oligosaccharide core, and confirmed by experiment.

Therefore by running a dextran ladder with unknown glycan mixtures under the same HPLC conditions, GU values can be determined for the peaks, enabling glycan structures to be assigned to the peaks.

In the following table: Man=mannose, Glc=glucose; A=antennary arms, G=galactose, F=fucose, B=bisecting N-acetylglucosamine, N=terminal N-acetylglucosamine, S=sialic acid; the columns headed H, N, F and S indicate the number of hexoses (i.e. mannose+galactose), N-acetylglucosamine, fucose and sialic acids, respectively, in each structure; MW indicates the molecular weight of the 2AB labelled structure; 'Gu calc.' is the GU value derived by calculation; 'Gu meas.' is the GU value obtained by experiment.

Oligosaccharide	Structure:	H	N	F	S	M.W. (2AB)	Gu calc.		Gu meas.		
Oligomannose-type N-linked Oligosaccharides:											
Oligomannose 9	Man-9	9	2	0	0		9.51		9.50		
Oligomannose 8	Man-8	8	2	0	0						
Oligomannose 8 D1, D3	Man-8 D1,D3	8	2	0	0						
Oligomannose 8 D1, D2	Man-8 D1,D2	8	2	0	0		8.83		8.81		
Oligomannose 8 D2, D3	Man-8 D2,D3	8	2	0	0						
Oligomannose 7	Man-7	7	2	0	0						
Oligomannose 7 D1	Man-7 D1	7	2	0	0		7.92		7.91		
Oligomannose 7 D2	Man-7 D2	7	2	0	0						
Oligomannose 7 D3	Man 7 D3	7	2	0	0						
Oligomannose 6	Man-6	6	2	0	0		7.07		7.06		
Oligomannose 5	Man-5	5	2	0	0		6.20		6.09		
Hybrid-type Native N-linked Oligosaccharides:											
Hybrid	Hybrid type				0						
	Glc3Man9				0						
	Glc3Man8				0						
	Glc3Man7				0		10.21				
	Glc3Man4				0		7.54				
Neutral Complex-type Native N-Linked Oligosaccharides:											
Asialo-, agalacto-, biantennary	A2G0 (NGA2)	3	4	0	0	1339.49	5.49		5.42-5.92		
	A2G0B (NGA2B)	3	5	0	0	1542.57	5.68		5.58		
	A2G0F(a1,3)(NGA2F)	3	4	1	0	1485.54			6.28		
Asialo-, agalacto-, biantennary, core-substituted with fucos	A2G0F(a1,6)(NGA2F)	3	4	1	0	1485.54	5.90		5.92-5.93		
	A2G0F2	3	4	2	0				6.72		
biantennary, core substituted with fucose and with bisectin	A2G0FB (NGA2FB)	3	5	1	0	1688.62	6.19		6.23-6.24		
	A2G1(1,3)	4	4	0	0	1501.54	6.50		6.50		
	A2G1(1,3)B	4	5	0	0	1704.64	6.69		6.68		
	A2G1(1,3)F	4	4	1	0	1647.60	6.91		6.80-6.82		
	A2G1(1,3)FB	4	5	1	0	1850.68	7.20		6.86-7.03		
	A2G1(1,6)	4	4	0	0	1501.54	6.38		6.38		
	A2G1(1,6)B	4	5	0	0	1704.64	6.57		6.60		
	A2G1(1,6)F	4	4	1	0	1647.50	6.79		6.68-6.70		
	A2G1(1,6)FB	4	5	1	0	1850.68	7.08		6.80		
	A2G1F2	4	4	2	0	1793.60	7.49				
	A2G1(1,3)FBF	4	5	2	0	1996.70	7.90		7.50-7.65		
	A2G1(1,6)FBF	4	5	2	0	1996.70	7.78		7.46-7.50		
Asialo-, galactosylated biantennary	A2G2 (NA2)	5	4	0	0	1663.40	7.15		7.16		
Asialo-, galactosylated biantennary with bisecting GlcNAc	A2G2B (NA2B)	5	5	0	0	1866.50	7.30		7.31		
Asialo-, galactosylated biantennary, core substituted with fu	A2G2F (NA2F)	5	4	1	0	1809.50	7.56		7.57		
	A2G2F2	5	4	2	0	1955.60	8.26				
	A2G2F2F	5	4	3	0	2101.70	8.96		8.67		
	A2G2F3	5	4	3	0	2101.70	9.66				
	A2G2F4	5	4	4	0	2247.80	10.36				
biantennary, core -substituted with fucose and with bisecti	A2G2FB (NA2FB)	5	5	1	0	2012.60	7.85		7.67-7.68		
	A2G2FBF	5	5	2	0				8.38		
	A2G2FBF2	5	5	3	0	2304.8	9.25		9.10-9.14		
Asialo-, agalacto-, triantennary	A3G0 (NGA3)	3	5	0	0	1542.5	5.91		5.91		
	A3G0B	3	6	0	0	1745.6	6.10		6.15		
	A3G0F	3	5	1	0	1688.6	6.32		6.36		
	A3G0FB	3	6	1	0	1891.7	6.61		6.68		
	A3G1FB	4	6	1	0	2053.7	7.46		7.50		
	A3G1FBF	4	6	2	0	2199.8	8.16		7.72		
	A3G2	5	5	0	0	1866.5	7.61		7.90		
	A3G2B	5	6	0	0	2069.6	7.80				
	A3G2F	5	5	1	0	2012.6	8.02		7.85		
	A3G2FB								8.03		
	A3G2FBF	5	6	2	0	2361.8	8.72		8.52		
	A3G2F2BF	5	6	3	0	2507.9	9.42		9.12		
	A3G2FF	5	5	2	0	2158.7	9.01				
Asialo-, galactosylated triantennary	A3G3 (NA3)	6	5	0	0	2028.5	8.65		8.35		
	A3G3B	6	6	0	0	2231.6	8.46				
	A3G3F	6	5	1	0	2174.6	8.87		8.74		
	A3G3F2	6	5	2	0	2320.7	9.57				
	A3G3FB	6	6	1	0	2377.7	9.16		8.89-9.14		
	A3G3FFB								9.32		
	A3G3F2BF	6	6	3	0	2669.9	10.56		10.02		
	A3G3F3BF	6	6	4	0	2816.0	11.26		10.72		
	A3G3FF2	6	5	3	0	2466.8	9.86		10.18		
Asialo-, agalacto-, tetraantennary	A4G0 (NGA4)	3	6	0	0	1745.6	6.54		6.54		
	A4G0B (NGA4B)	3	7	0	0	1948.7	6.73		6.53		
	A4G0F	3	6	1	0	1891.7	6.95		6.82		
	A4G0FB	3	7	1	0	2094.8	7.24		6.90		
	A4G1	4	6	0	0	1907.6	7.39		7.48		
	A4G1B	4	7	0	0	2110.7	7.58				
	A4G1F	4	6	1	0	2053.7	7.80				
	A4G1FB	4	7	1	0	2256.8	8.09		7.85		
	A4G1FBF	4	7	2	0		8.79		8.24		
	A4G2	4	7	0	0		8.24				
	A4G2B	5	7	0	0		8.43				
	A4G2F	5	6	1	0		8.65		8.58		
	A4G2FB	5	8	0	0		8.94				
	A4G2FBF	5	8	2	0		9.64		9.04		

Oligosaccharide	Structure:	H	N	F	S	M.W. (2AB)	Gu calc.		Gu meas.		
	A4G2FBF2	5	8	3	0		10.34		9.74		
	A4G3	6	6	0	0		9.09				
	A4G3B	6	7	0	0		9.50				
	A4G3F	6	6	1	0		9.28		9.31		
	A4G3F3	6	6	3	0		11.19		11.36		
	A4G3FB	6	7	1	0		9.79		9.37/9.98		
	A4G3FBF	6	7	2	0		10.49		10.76		
	A4G3FBF3	6	7	4	0		11.89				
Asialo-, galactosylated tetraantennary	A4G4 (NA4)	7	6	0	0		10.13		9.68		
	A4G4B	7	7	0	0		9.94				
	A4G4F	7	6	1	0		10.35		9.96		
	A4G4FB	7	7	1	0		10.64		10.36		
	A4G4FBF	7	7	2	0		11.34		10.64		
	A4G4FBF2	7	7	3	0		12.04		11.66		
	A4G4FBF3	7	7	4	0		11.34				
	A4G4FF	7	6	2	0		11.05		10.81		
Native N-Linked Oligosaccharide Core Products:	M2N2	2	2	0	0				3.46		
	M2N2F(a1-6)	2	2	1	0				3.97		
	M2N2F(a1-3)	2	2	1	0				4.29		
	M2N2F2	2	2	2	0				4.76		
trimannosyl core	M3N2	3	2	0	0		4.42		4.38-4.44		
trimannosyl core, 1-6 fucose on reducing end GlcNAc	M3N2F(a1-6)	3	2	1	0		4.91		4.86-4.97		
trimannosyl core, 1-3 fucose on reducing end GlcNAc	M3N2F(a1-3)	3	2	1	0		5.32		5.29-5.32		
	M3N2F2	3	2	2	0		5.32		5.68-5.74		
	M3N3	3	3	0	0				5.00		
	M3N3F(a1-6)	3	3	1	0				5.32		
	M3N3										
6-a-fucosyl chitobiose	N2F	0	2	1	0				2.30		
	N2F2						2.80				
6-a-fucosyl GlcNAc	NF	0	1	1	0				1.69		
Chitobiose	NN	0	2	0	0				1.73		
4'-b-mannosyl chitobiose	MNN	1	2	0	0		2.65		2.66		
4'-b-mannosyl 6-a-fucosyl chitobiose	MNNF	1	2	1	0		3.17		3.18		
Conserved tri-mannosyl core, substituted with fucose and x	M3N2FX	3	2	1	0						
Sialylated structures:											
	A2G2S(3) (A1)	5	4	0	1				7.49	7.57	
	A2G2S(6) (A1)	5	4	0	1				7.89	7.98	
	A2G2S2(3)2 (A2)	5	4	0	2				7.72		
	A2G2S2(6)2 (A2)	5	4	0	2				8.65	8.80	
	A2G2S2(6)(3) (A2)	5	4	0	2				8.28	8.42	
	A2G2FS(6)	5	4	1	1				8.27	8.32	
	A2G2FS(3)	5	4	1	1				8.10	8.26	
	A2G2FS2 (A2F)	5	4	1	2				8.82		
	A3G2S(3)										
	A3G3S(3)	6	5	0	1				8.56	8.64	
	A3G3S(6)	6	5	0	1				8.94	9.03	
	A3G3S2(3)2	6	5	0	2				8.86	9.00	
	A3G3S2(6)2	6	5	0	2				9.61	9.77	
	A3G3S2(6)(3)	6	5	0	2				9.22	9.37	
	A3G3S3(6)3	6	5	0	3				10.25		
	A3G3S3(3)3 (A3)	6	5	0	3				9.16		
	A3G3S3(6)(3)2	6	5	0	3				9.54	9.78	
	A3G3S3(6)2(3)	6	5	0	3				9.89	10.15	
	A3G3S4(6)2(3)2[$]	6	5	0	4				9.99	10.28	
	A3G3S4(6)3(3)1	6	5	0	4				10.33	10.63	
	[$] 1xS(6) attached to GlcNAc. Antenna on Manb1-4 arm is Galb1-3GlcNAc										

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