Antibody specificity in neurological disease

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By

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Abstract

The study of antigen-specific intrathecal oligoclonal bands is well established and a number of methods have been used to demonstrate that the relative affinity of the antibody produced in multiple sclerosis is low, and in encephalitis, high. A method colloquially known as Eastern blotting was developed whereby relative affinity of individual clones, rather than total antibody, could be studied and quantified by antigen immunoblotting and investigation of a digitised blot using image-manipulating software. This method was used to show that the pixel density of a band in CSF was significantly greater than the same band in serum in a patient with SSPE, and was thus of intrathecal origin. Eastern blotting was then used on a series of samples from a patient with herpes encephalitis to demonstrate that affinity maturation of the immune response had occurred intrathecally. The method was used qualitatively to investigate a proposal that Acinetobacter sp. infection could be the primary cause of multiple sclerosis: no evidence could be found to support the hypothesis. Another suggested cause of multiple sclerosis, Chlamyphila pneumoniae, was studied using a variety of methods including Western blotting. Again, there was no evidence to support the hypothesis. During the project, an unexpected effect of high-strength thiocyanate was revealed, and limited study of this suggested that thiocyanate had an effect on IgG, possibly related to the age of the sample.
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<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
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<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
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<tr>
<td>BCR</td>
<td>B cell receptor</td>
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<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSE</td>
<td>Bovine spongiform encephalopathy</td>
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<tr>
<td>C region</td>
<td>Constant region</td>
</tr>
<tr>
<td>CALLA</td>
<td>Common acute lymphoblastic leukaemia antigen</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CMLD</td>
<td>Carboxymuconolactone decarboxylase</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CRS</td>
<td>Congenital rubella syndrome</td>
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<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<td>CVA</td>
<td>Cerebrovascular accident</td>
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<tr>
<td>D region</td>
<td>Diversity region</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>EB</td>
<td>Eastern blotting</td>
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<td>EBF</td>
<td>Early B cell factor</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>ELONA</td>
<td>Enzyme-linked oligonucleotide assay</td>
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<tr>
<td>GLM</td>
<td>General linear model</td>
</tr>
<tr>
<td>HEEO</td>
<td>High electroendosmosis (agarose)</td>
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<tr>
<td>HHV</td>
<td>Human herpes virus</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>HSA</td>
<td>Human serum albumin</td>
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<tr>
<td>HSE</td>
<td>Herpes simplex encephalitis</td>
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<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>ICD</td>
<td>International Classification of Diseases</td>
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<td>IEF</td>
<td>Isoelectric focusing</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
</tr>
<tr>
<td>IgD</td>
<td>Immunoglobulin D</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IGH</td>
<td>Immunoglobulin heavy (chain cluster)</td>
</tr>
<tr>
<td>IGK</td>
<td>Immunoglobulin kappa (light chain cluster)</td>
</tr>
<tr>
<td>IGL</td>
<td>Immunoglobulin lambda (light chain cluster)</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>J region</td>
<td>Joining region</td>
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<tr>
<td>LDS</td>
<td>Lithium dodecyl sulphate</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>MEEO</td>
<td>Medium electroendosmosis (agarose)</td>
</tr>
<tr>
<td>MES</td>
<td>2-[N-Morpholono]ethanesulphonic acid</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MIF</td>
<td>Microimmunofluorescence</td>
</tr>
<tr>
<td>MOG</td>
<td>Myelin oligodendrocyte glycoprotein</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>NHNN</td>
<td>National Hospital for Neurology and Neurosurgery</td>
</tr>
<tr>
<td>NS</td>
<td>Not significant</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OPD</td>
<td>o-phenylenediamine</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PCA</td>
<td>Plasma cell antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
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<td>Polymerase chain reaction enzyme-linked oligonucleotide assay</td>
</tr>
<tr>
<td>PCR-RFLP</td>
<td>Polymerase chain reaction restriction fragment length polymorphism</td>
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<tr>
<td>PHSC</td>
<td>Pluripotent haemopoietic stem cell</td>
</tr>
<tr>
<td>PI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear neutrophil</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidifluoride</td>
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<td>RAG</td>
<td>Rabbit anti-goat</td>
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<tr>
<td>RIA</td>
<td>Radio-immuno assay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SAR</td>
<td>Swine anti-rabbit</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SSPE</td>
<td>Subacute sclerosing panencephalitis</td>
</tr>
<tr>
<td>Tdt</td>
<td>Terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TIFF</td>
<td>Tag image file format</td>
</tr>
<tr>
<td>TSE</td>
<td>Transmissible spongiform encephalopathy</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V region</td>
<td>Variable region</td>
</tr>
<tr>
<td>VDRL</td>
<td>Venereal Disease Research Laboratory slide test</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
</tbody>
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1.0 Introduction

1.1 B Cell Ontogeny

1.1.1 Introduction to B cell Ontogeny

The immune system is a combination of specialised cells and molecules that mediates two different types of response to antigenic stimulus. The innate, or natural, response is always the same regardless of how many times a particular stimulus is met but the acquired, or adaptive, response improves on repeated exposures.

Phagocytes, natural killer cells, inflammatory mediators, acute phase proteins, some cytokines and complement mainly mediate the innate response. The acquired response is mediated by B and T lymphocytes and immunoglobulins secreted by the B cells. The immune response to any given stimulus is usually a combination of both.

Both B cells and T cells derive from a common lymphoid progenitor (CLP), itself derived from a pluripotent haemopoietic stem cell, B cells accounting for 5 to 15% of the circulating lymphoid cell population.

In the chicken, primary B cell development occurs in the bursa of Fabricius, hence the title B or bursal lymphocytes. In mammals, it begins in the foetal liver at approximately eight to nine weeks’ gestation after which the bone marrow assumes B cell production, where it remains for life.

In brief, the stem cell first becomes a pre-pro-B cell, at which point it is committed to becoming a B cell. It then matures successively into a pro-B, pre-B, immature B, transitional B and mature B cell (Figure 1.1). Each stage can be classified according to the surface and cytoplasmic markers expressed. Maturation is characterised by the rearrangement and expression of immunoglobulin genes.
Figure 1.1 B lymphocyte differentiation.
1.1.2 Stem cell to pre-pro-B cell

The pluripotent haemopoietic stem cell (PHSC) is thought to give rise to common myeloid- and lymphoid-restricted progenitors (Weissman, 2000), the latter giving rise to both B and T lymphocytes. In the bone marrow, the stem cell lies close to the endosteal cell layer at the edge of the bone marrow cavity.

There are three known essential transcription factors which initiate B lymphopoiesis from the PHSC: E2A and early B cell factor (EBF) (Rolink et al., 2001) do not determine B cell commitment unlike Pax5 (Nutt et al., 1999), although it is not known whether or not the commitment is reversible.

The pre-pro-B cell can now be distinguished from its CLP precursor by the cell surface markers, cluster of differentiation (CD) 43 and CD45R.

1.1.3 The pro-B cell to mature B cell

Once the stem cell has matured into the pro-B cell it expresses the CD10 surface antigen, the common acute lymphoblastic leukaemia antigen (CALLA), which is specific for pre-B cells. The pro-B cell also differs from its precursors because immunoglobulin gene recombination begins and they express a forerunner of the B cell receptor (BCR).

The immunoglobulin molecule consists of two heavy chains joined by a disulphide bridge, and two light chains, each attached to one of the heavy chains through a disulphide bridge. Where the light and heavy chains run parallel they form two antigen-binding sites with their variable domains (Edelman, 1973). Each chain has a constant (C) region, which determines the chain type or class. In order to produce antibodies with different antigen specificities an individual must therefore have the genetic “ability” to produce a large number of variable (V) domain sequences. Dreyer and
Bennett (1965) proposed that separate "genes", which underwent rearrangement, coded for V and C regions. It has been estimated that about $10^{15}$ variable regions can be produced from fewer than 400 genes (Tonegawa, 1983). The use of recombinant deoxyribonucleic acid (DNA) technology and restriction enzymes showed that genes for immunoglobulins, unlike most genes, were not intact and functional but were broken up along the chromosome into gene segments (Hozumi and Tonegawa, 1976).

The genetic components for immunoglobulins are found on three chromosomes; chromosome 14 for the immunoglobulin heavy chain (IGH) cluster, chromosome 2 for the immunoglobulin kappa (IGK) (κ or kappa light chain) cluster and chromosome 22 for the immunoglobulin lambda (IGL) (λ or lambda light chain) cluster. Each cluster contains gene segments coding for V, joining (J) and C segments. The IGH cluster also contains D gene segments coding for diversity (D), (Delves and Roitt, 2000).

The first event in gene rearrangement is the recombination of a D and J segment from the IGH cluster on both copies of chromosome 14 followed by the recombination of one of these DJ segments with a V segment. If this VDJ recombination is non-productive then a VDJ recombination occurs on the other DJ segment. Once a productive VDJ recombination has been generated then it is joined to a C segment. There are five of these corresponding to the five classes of antibody, G, A, M, D and E. The light chains are similarly coded, though without the D segment, and have only one C exon in a cluster, hence the production of either λ or κ light chains. Thus, VDJC (for heavy chain) and VJC (for light chain) recombination form a linear coding unit for that particular chain. Immunoglobulins G and A and λ light chains all have subclasses. Inaccuracies in the splicing of the VDJ junctions can lead to slight variations in the nucleotidie sequence and terminal deoxynucleotidyl transferase (Tdt) may also insert
additional nucleotides thus further increasing diversity (Schatz et al, 1992) and leading to a possibly unique immunoglobulin molecule (Figure 1.2).

The pre-B cell can now synthesise M heavy chains that are linked to a "pseudo light chain" and presented as a cell surface surrogate "immunoglobulin M (IgM)" receptor. This is essential for further differentiation as the receptor is signalled to suppress heavy chain gene rearrangement on the sister chromatid: a process called allelic exclusion. Gene rearrangements now occur in the κ light chain loci until a productive rearrangement is found. The cell can now synthesise conventional surface IgM which effects allelic exclusion on the light chain genes thus ensuring the progeny of the cell will continue to make the same immunoglobulin chains, the phenomenon called clonal restriction.

The cell is now expressing surface IgM, possibly along with immunoglobulin A (IgA), immunoglobulin G (IgG) and immunoglobulin D (IgD), which primes the immature B cell for antigen response. Tdt and CD10 have been lost as surface antigens and the cell now expresses CDs 19, 20, 21, 23 and 45R alongside Major Histocompatibility Complex (MHC) II.

The next stage in the development of the B cell is the first exposure to antigen. To be part of the immune response the B cell must express a receptor that will bind the antigen, the process of clonal selection. The stronger the binding, the more likely it is that the B cell will survive and proliferate.

At this point, it is worth remarking on the two sub-populations of B cells, B1 and B2. B1 cells develop in the foetus, are located mainly in the peritoneal cavity (Su and Tarakhovsky, 2000) and usually express CD5 as a surface marker (B1a), but not always (B1b). They produce polyreactive IgM ("natural" antibodies) of low affinity (Hayakawa et al, 1999) that are often self-reactive (Hayakawa et al, 1984). B1 cells are positively
selected by self-antigens (Hayakawa et al, 1999) and are a self-renewing population that may be controlled by secreted IgM (Hayakawa and Hardy, 2000).

The B2 cells that develop later in the bone marrow, few of which express CD5, co-express IgM and IgD and it is these that proliferate after antigenic stimulus. They do not have autoreactivity (Cornall et al, 1995).

### 1.1.4 Affinity Maturation

After antigenic stimulation, B cells mature further into either memory cells or plasma cells, in an area within the lymph node called the germinal centre. Once bonding with antigen has activated the B cell, it becomes a blast cell, of which a very small number form a germinal centre. Surrounded by resting B cells and sustained by follicular dendritic cells, B cells proliferate in the dark zone of the germinal centre. Here they undergo somatic hypermutation of the variable region genes, which “fine tunes” specificity. The proliferating cells migrate to the basal light zone where they stop proliferating and begin to upregulate expression of surface immunoglobulin. In here, there is extensive positive selection of high-affinity, antigen-specific B cells thought to be driven by antigen-antibody complexes deposited on the follicular dendritic cells. However, a recent study in transgenic mice unable to secrete IgM showed that the lack of immune complexes did not prevent a normal immune response (Hannum et al, 2000). The blast cell expresses CD10 again but only during proliferation. B cells not positively selected at this stage apoptose and are subsequently phagocytosed by macrophages, becoming “tingible bodies” within the macrophage. B cells that are positively selected migrate to the apical light zone where isotype switching occurs. The genetic mechanism underlying this is the recombination of the VJ segment with a different C segment.
Actions of TdT give rise to further mutations and increase diversity.

Following B cell activation both light and heavy chain V regions mutate 10000 quicker than background mutation rate thus further increasing diversity.

Figure 1.2 Generation of antibody diversity.
Although the antibody specificity remains the same, (as coded for by the V region), the different heavy chain isotype can give the immunoglobulin different properties with regard to complement binding, receptor interaction and immunoglobulin transfer across membranes.

As the antigen concentration decreases only those cells with high affinity receptors will be sustained. Those cells now further differentiate into either memory B cells or plasma cells under the influence of CD40 or soluble interleukin (IL)-1-α together with CD23, respectively. Memory cells enable a faster and better secondary immune response after re-encountering the antigen and commonly express IgA or IgG as a surface antigen. Plasma cells have no surface immunoglobulin and express different surface markers from memory cells. Plasma cells express CD38, which is initially lost during the transmission from pre-B cell to immature B cell and plasma cell antigen 1 (PCA-1), a unique antigen found only on the plasma cell. Plasma cells mature away from the germinal centre and have a well-defined endoplasmic reticulum and Golgi apparatus with which they can synthesise and secrete immunoglobulin.

1.2 Antibody Affinity and Avidity

Antibody affinity can be defined as the strength of the binding between an antibody and an antigen and is a combination of hydrogen bonds, electrostatic interactions, van der Waals forces and hydrophobic bonds. If there is, stoichiometrically, a good fit between the binding site of the antibody and the antigenic determinant, or epitope, then many attractive forces are created and few repulsive forces so there is antibody-antigen binding of high affinity. However, if the fit is poor then repulsive forces dominate so the antibody will be of low affinity for that epitope. When the fit is so poor that high repulsive forces are generated, there may be no attraction and thus no affinity.
Affinity relates specifically to a single antigenic determinant binding with a single combining site and is not the same as avidity, which is the overall strength of antibody-antigen binding, in which either antibody or antigen or both can be multivalent. If all binding sites on the antibody can combine with the antigen the avidity will be greater than the sum of affinities because all the interactions must be simultaneously dissociated.

The ability of an antibody to recognise an antigen lies in the shape of the epitope, a combination of stearicity and charge, rather than just its chemical properties. Thus antibodies can be very specific and can distinguish subtle differences in epitope conformation. However, two or more antigens may share an epitope, in which case antibodies raised primarily against one will cross-react with the other. This phenomenon is known as molecular mimicry and can lead to autoimmune disease. A classic example of a bacterium causing molecular mimicry is *Campylobacter jejuni*. Following infection with Campylobacter, antibodies can be raised against the lipopolysaccharides of the organism, which then cross-react with glycolipids in the myelin sheath, leading to Guillain-Barre syndrome or Miller Fisher syndrome (Willison and Yuki, 2002).

The affinity and avidity of an antibody are clearly of importance with regards to its physiological function. Immune complexes of antigen with high-affinity antibody are removed more quickly from the circulation than those involving low-affinity antibodies because they are more efficient at activating the complement pathways and stimulating neutrophils to release lysosomal enzymes (Marzocchi-Machado *et al*, 1999).

### 1.3 Chaotropism

The word ‘chaotrope’ derives from the Greek: Chaos, meaning disorder or confusion, and trope, meaning a turning. Thus, a chaotropic molecule acts by disrupting the chemical system into which it is introduced.
The reversible nature of the antibody-antigen bond is the chemical basis on which chaotropic molecules, such as sodium thiocyanate (NaSCN), work. In solution, the thiocyanate ion, SCN-, is small and highly charged and so can infiltrate the antibody-antigen interface. If the antibody-antigen bond is sufficiently “tight”, i.e. the antibody has high affinity for the antigen, then a relatively high concentration of the chaotrope would be required to exert sufficient “pressure” to disrupt the bond. The less “tight” the bond the progressively lower concentration of chaotrope would be needed to disrupt the binding and therefore the lower the affinity of the antibody for that antigen.

As all the forces involved in antibody-antigen binding are reversible, then the antibody-antigen bond must also be completely reversible and therefore subject to the Law of Mass Action which defines the behaviour of a chemical equilibrium of the nature:

\[ A + B \rightleftharpoons C + D \]

In terms of antibody-antigen reactions it can be written as

\[ Ab + Ag \rightleftharpoons AbAg \]

where \( Ab = \) antibody, \( Ag = \) antigen, \( AbAg = \) antibody-antigen complex, \( \rightleftharpoons = \) reversible reaction.

If the antibody has a high affinity for the antigen the equilibrium will lie over to the right. The affinity of the antigen-antibody bond can be defined with the equilibrium constant, \( Ka \), of the association reaction:

\[ Ka = \frac{[AbAg]}{[Ab][Ag]} \]

where \([ ]\) represents concentration.

The disassociation reaction and its equilibrium constant, \( Kd \), is the inverse

\[ Kd = \frac{[Ab][Ag]}{[AbAg]} \]

and its value is the reciprocal of \( Ka \).

The term “affinity” strictly refers to the binding of the antibody to a single antigenic determinant whereas in practice antigens are often multivalent and react with polyclonal
serum derived from either a patient or a commercial source. This interaction, the sum of affinities, is called ‘avidity’ or ‘functional affinity’. With a multivalent antigen and a polyclonal serum numerous antibodies can simultaneously bind the antigen so that when one of the antigen-antibody bonds dissociates the antigen can still be held by the others. This means that antigen-antibody binding is greater than the sum of the individual affinities, as the Ka values of each bond can be multiplied to obtain the final Kavid, the “avidity constant” or “functional affinity constant”.

In practice, the term “affinity” is often used where “avidity” is the correct term, even within peer-review journals.

The effects of salts of the thiocyanate ion on antibody-antigen interactions have been studied for many years. In 1978, Shimizu et al used potassium thiocyanate to elute antidinitrophenyl antibodies from an immunoadsorbent. They found that the mean affinity of their elutions increased along with the molarity of the thiocyanate solution used to elute them. Pullen et al (1986) investigated the avidity of human anti-rubella antibodies post-vaccination in an enzyme-linked immunosorbent assay (ELISA). Using increasing concentrations of thiocyanate they measured resistance to elution as an indicator of avidity and so generated an affinity index to compare different sera. The index itself was defined as the concentration of thiocyanate that produced a 50% reduction in absorbance. Luxton and Thompson (1990) used thiocyanate elution followed by ELISA to look at the distribution of antibody affinity in paired serum and cerebrospinal fluid (CSF) samples in multiple sclerosis (MS) and viral encephalitis. Using thiocyanate concentrations up to 5M, at 0.5M intervals, they consistently showed that CSF IgG against viral antigens, (measles, herpes, varicella, rubella, toxoplasma, mumps and cytomegalovirus (CMV)), was of lower affinity in multiple sclerosis patients, (n=19), than in those patients with actual viral encephalitis, (subacute sclerosing panencephalitis (SSPE), n = 4; herpes simplex, n = 3 and varicella, n=3). Luxton and Thompson
calculated their results as relative affinities using absorbance with and without thiocyanate (the control), expressed as a percentage. Further work by Luxton (1995) refined the relative affinity calculation into an affinity ratio (of high to low affinity antibody) to distinguish MS from encephalitis. This confirmed their previous observation that CSF IgG was of low affinity in MS, but of high affinity against the causative organism in encephalitis.

In 1988, Yeh and Krolick undertook to determine the avidities of clonotypes of IgG generated in experimental autoimmune myasthenia gravis by blotting focused samples onto antigen-coated membrane. As a method of assessing the relative avidities of the IgG subtypes they blotted onto nitrocellulose membranes coated with a sixteen-fold range of antigen concentrations. The concentration at which a band first appeared was used as a qualitative measure of the avidity of the band. A densitometric scan was taken of the results but used only to demonstrate the presence or absence of peaks.

The principle of isoelectric focusing (IEF) followed by blotting onto antigen-coated membrane was established by Moyle et al (1984). However, few studies incorporated the incubation of the focused, blotted membrane with chaotropic agents to establish the avidities of the antigen-specific antibodies. Marcolino et al (2000) used avidity to distinguish between the acute and chronic phases of toxoplasmosis. They separated a preparation of *Toxoplasma gondii* by PAGE and blotted onto nitrocellulose before incubating with serum samples and detecting the IgG. They probed for avidity with 6M and 8M urea. The avidity immunoblotting was used to confirm the results of an avidity ELISA whereby an avidity index was calculated based on absorbances with and without incubation with urea. Serum dilutions for the immunoblot were based on those that gave the smallest index on the ELISA. This method was also used by Nedeljkovic et al (2001) when investigating affinity maturation of IgG to rubella virus proteins.
Thiocyanate elution was therefore seen as a simple and reliable method of estimating relative antibody avidities. When incorporated into an ELISA format the resulting data could be analysed quantitatively and it appeared robust enough to be used with a variety of antigens and antibody concentrations. In 1992, Gray and Shaw reported artefactual increase of bound antibody with an adaptation of the ELISA method of Pullen et al (1986). Finding the phenomenon in both polyclonal and monoclonal antibody preparations they proposed that exposure to thiocyanate induced a rearrangement in the primary antibody molecule that exposed more reactive epitopes to the secondary antibody in the assay and hence produced the artefactual increase. Their subsequent investigations into the possible effects of thiocyanate failed to produce any definitive answer; rather, they highlighted a number of possible factors involved, mainly the multiple binding of IgM and possible Fab reattachment at adjacent epitopes. They reiterated Lews (1984) work on the effect of epitope density and also proposed that specificity of the secondary antibody may have a part to play. McMahon and Kennedy (2000) reported that exposure to low pH (during affinity purification) changed a monoreactive to a polyreactive antibody against self-antigens, an interesting observation since acids can be used as chaotropes. They concluded that polyreactivity, in this case, was an acquired artefact after limited denaturation and not an “unmasking” of a physiological property of immunoglobulin. This same enhancement of antibody activity to both self and non-self antigens was also found by Bouvet et al (2001a) using acid buffer, 1.3M NaSCN and 6M urea. However, they observed that the dissociation constant of the IgG studied did not change after exposure to chaotrope and hence there was no increase of avidity. Rather, they postulated that basic polyreactive antibody structures were exposed by dissociating agents and could be of physiological significance. After rejecting the conclusions of McMahon and Kennedy (2000), Bouvet
(2001b) further elucidated his ideas that the polyreactive autoantibodies were
evolutionarily conserved.

1.4 Intrathecal Synthesis of Immunoglobulin G

The term ‘intrathecal ‘ derives from the word ‘thecum’, which was defined as the
cerebrospinal fluid, spinal cord and spinal roots compartmentalised by a surrounding
membrane of dura mater (uscneurosurgery.com). In medical science, intrathecal is now
accepted as meaning within the meninges, the three connective tissue membranes that
enclose the brain and spinal cord, of which the dura mater forms the outer layer. Thus,
IgG that has an intrathecal origin is synthesised within the central nervous system
(CNS) as opposed to that of systemic origin. Normally the homogenous, polyclonal
background seen in CSF is largely derived from serum immunoglobulins “leaking”
across the blood-brain barrier (BBB) in low concentrations. However, when there is
also an oligoclonal pattern an underlying pathology is likely (Figure 1.3). Oligoclonal
bands (OCBs) are visualised in the laboratory as discrete bands clearly visible against
the polyclonal background and are immunoglobulin secreted by a limited number of
clones of plasma cells. Due to the low background concentration of IgG, OCBs need
only be of a low concentration themselves to be easily visible.

Conventionally, OCB patterns are reported in five different ways (Table 1.1) including
patterns where monoclonal IgG is detected (Andersson et al, 1994). Laboratory
investigation of OCBs ideally requires contemporaneous serum/plasma and CSF so that
the two can be compared with regard to any bands that are seen.
### 1.4.1 Oligoclonal band patterns

#### Table 1.1 Oligoclonal band pattern reporting.

<table>
<thead>
<tr>
<th>Pattern</th>
<th>Abbreviation</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>-</td>
<td>No bands in serum or CSF</td>
</tr>
<tr>
<td>Positive</td>
<td>+</td>
<td>Bands in CSF only</td>
</tr>
<tr>
<td>Mirror</td>
<td>*</td>
<td>Same bands in serum and CSF</td>
</tr>
<tr>
<td>Greater than</td>
<td>&gt;&gt; or &gt;</td>
<td>More bands in CSF than serum</td>
</tr>
<tr>
<td>Paraprotein</td>
<td>P</td>
<td>Paraprotein</td>
</tr>
</tbody>
</table>

As reported in Neuroimmunology laboratory, National Hospital for Neurology and Neurosurgery (Andersson et al, 1994).

The presence of CSF OCBs of IgG indicates only that there is a humoral immune response. If the immune response is being conducted in the serum, then by the normal processes of ultrafiltration that generate CSF those bands can be seen in the CSF, underlining the importance of interpreting both serum and CSF together. Beyond this, however, OCBs are not of themselves very specific as a large number of pathologies result in OCBs. Anything that generates an immune response such as infection, inflammation and neoplasia may result in OCBs so it is important to look at their distribution between serum and CSF. If the OCB pattern is identical in serum and CSF then this indicates a systemic origin for immune activation with IgG crossing the blood-brain barrier easily (Thompson, 1988). In patients where there is intrathecal synthesis of IgG there will be OCBs either in the CSF only, or if there are OCBs in the serum, there will be more bands in the CSF. In the former case there is only intrathecal synthesis and in the latter, intrathecal synthesis occurs in addition to a systemic response.
KEY: S = Serum, C = CSF. The blot number lies at the anodal edge.

1. Serum negative, CSF negative (S-, C-):
   No evidence of intrathecal IgG synthesis (normal pattern).

2. Serum negative, CSF positive (S-, C+):
   Oligoclonal bands present in CSF only. Indicates intrathecal synthesis of IgG.

3. Serum positive, CSF positive (S+, C+):
   Identical pattern of bands in both serum and CSF. Indicates systemic synthesis of IgG "leaking" into the CSF.

4. Serum positive, CSF greater (S+, C>>) or (S+, C>>):
   Oligoclonal bands present in both serum and CSF with extra bands in CSF. Indicates both intrathecal and systemic IgG synthesis.

5. Serum positive, CSF positive (Sm, Cm):
   Abnormal identical pattern of bands in both serum and CSF, usually seen with a monoclonal protein. Indicates peripheral IgG synthesis "leaking" into the CSF.

Figure 1.3 Oligoclonal band patterns.

Images from www.ii.bham.ac.uk/clinicalimmunology/Neuroimmunology/IEF.htm
IEF also reveals patterns associated with monoclonal proteins. The appearance of the bands is very distinctive, consisting of an evenly-spaced "ladder" where the bands diminish in intensity towards the anode.

1.4.2 Viral encephalitis

There are a number of viruses that are more commonly neuroinvasive and neurotropic. Herpes simplex virus (HSV), measles, mumps, rubella, varicella-zoster, Epstein-Barr (EBV) (this is not a common cause of encephalitis; to the contrary, encephalitis has not been reported with EBV), Human herpes virus (HHV) 6 and 7 and enteroviruses are the commonest causes of acute encephalitis in the United Kingdom amongst individuals with no immune compromise (Chaudhuri and Kennedy, 2002). There are three ways that viruses can enter the CNS: directly through the nerves (neurally), through the nasal mucosa (olfactory) or via the bloodstream (haematogenously). Generally, viruses that cause encephalitis are common parasites of humans, so viral encephalitis is an uncommon complication of systemic infection. Some viruses rarely infect the CNS but give rise to significant pathologies when they do whereas others, such as those mentioned above, are common and trivial infections until they infect the CNS, when they become more serious.

Laboratory investigation of the CSF typically reveals a raised white cell count, with over 5 lymphocytes/mm³ (Davis, 2000), normal or slightly raised total protein and normal glucose.

1.4.2.1 Herpes simplex encephalitis

Herpes simplex encephalitis (HSE) is the most common viral encephalitis in immunocompetent patients and is either a primary infection, or a reactivation (Yamada et al, 2003) of a prior infection or reinfection (Whitley et al, 1982). It afflicts 1 to 4 per
million annually (Lipkin, 1997) and approximately a third of cases are in the under-20 and half in the over-50 age groups. Mortality of untreated cases is 70% with only 2.5% of survivors recovering fully (Whitley and Gnann, 2002). In adults HSV2 usually causes genital herpes, with encephalitis being a very rare complication. Consequently, neonatal HSE due to HSV2 transmitted during childbirth accounts for 80% of those cases, but only 10% of non-neonatal cases (Aurelius et al, 1993; Dennett et al, 1997). It has a rapid onset with no specific clinical symptom but once a diagnosis is made, often on the basis of CSF polymerase chain reaction (PCR), it can be treated successfully with acyclovir (Chaudhuri and Kennedy, 2002). However, therapy needs to begin as soon as herpes simplex infection is suspected (Wutzler, 1997). This was underlined by a study on seven patients (Bell et al, 2003), which showed that severity of disease and survival in initially untreated patients was dependent on a strong intrathecal response. The virus is thought to spread, in reactivated form, from cranial nerve ganglia into the brain (Esiri and Tomlinson, 1984), which may be aided by stress, trauma and immunosuppression. CSF analysis may also show a high erythrocyte count, over 500 cells/mm³, if HSE is necrotising or haemorrhagic, and this needs to be distinguished from traumatic tap. The use of PCR assays for herpetic viral DNA (Rowley et al, 1990) is now a common method of diagnosing HSE.

1.4.2.2 Measles encephalitis

The measles virus, a ribonucleic acid (RNA) virus belonging to a subgroup of paramyxoviruses, enters through the upper respiratory tract and may reach the brain by infection of cerebral endothelial cells (Kirk et al, 1991) or circulating inflammatory cells (McQuaid et al, 1993). Measles virus does not appear to be spread by extracellular virus or cellular fusion as it is in non-neuronal cells and a trans-synaptic mechanism has recently been proposed (Lawrence et al, 2000).
**1.4.2.2.1 Subacute sclerosing panencephalitis**

SSPE is a rare, usually fatal, chronic CNS infection affecting less than 1 per million of children with measles, that occurs years after the original acute infection and is caused by defective measles virus (Modlin et al, 1977; Modlin et al, 1979). Measles virus is plentiful in brain cells of SSPE patients, who are always immunocompetent, and so is seen as an opportunistic infection. There are high titres of anti-measles antibodies in serum and CSF of SSPE patients (Connolly et al, 1967), yet this does not control the infection (Metha et al, 1994). Studies show the original infection was at a very young age, as genotypes recovered from post mortem SSPE brain corresponded to those present at the time of infection (Jin et al, 2001). Astrocytes, microvascular endothelial cells, neurons and oligodendrocytes have all been found to be infected in cases of SSPE (Allen et al, 1996) although there is no direct evidence of CNS involvement during the acute measles infection. Indirect evidence from abnormal electroencephalographs and CSF pleocytosis (Hanninen et al, 1980) in approximately a third of acute measles infections suggest that the virus may enter the CSF at this point. Rammohan et al (1982) reported measles infection and subsequent SSPE in a patient who had been immunised. They then demonstrated (Rammohan et al, 1981; 1983) in rodents that the immature immune system of newborns would allow persistent CNS measles infection. Studies on mice unable to present antigen (Urbanska et al, 1997) suggested that measles virus spread through neurons that were targeted by cytotoxic T lymphocytes. Raised levels of the antiviral type 1 IFN in SSPE CSF also suggest presence of established virus (Joncas et al, 1976) although wild-type measles virus has been shown to be less efficient at inducing type 1 IFN than vaccine strains in peripheral human lymphocytes (Naniche et al, 2000). Persistence in the CSF may be explained by the presence of anti-measles antibody keeping levels of infectious virus low. The consequence of this is that mutations accrue in the viral genome resulting in loss of the ability to assemble
infectious virus (Cattaneo et al., 1988). The subsequent accumulation of viral products within cells may then lead to cell death (Ikeda et al., 1995) and sometimes demyelination. Some demyelination may also occur due to cell-mediated mechanisms (Nagano et al., 1994). Although it is difficult to isolate the virus from post mortem brain tissue it has been successfully done and the strain was still highly neurovirulent, even in vitro (Ito et al., 2002).

1.4.2.3 Mumps encephalitis

Aseptic meningitis as a consequence of mumps virus infection makes up approximately 90% of cases of acute mumps with CNS involvement (Butman, 1998). Principally an infection of childhood, like measles, the pathologic mechanism of mumps encephalitis is uncertain. Direct viral invasion can occur early in the disease whereas later, post-infectious encephalitis can involve autoimmune mechanisms leading to inflammation and demyelination.

1.4.2.4 Varicella zoster encephalitis

Infection with the exclusively human varicella-zoster virus, an α-herpes virus, usually leads to chickenpox (varicella), most commonly in childhood. The virus then becomes latent within the neurons of the cranial and spinal ganglia, occasionally reactivating as shingles (zoster). Very rarely does it become neuroinfectious (Barnes and Whitley, 1986) but encephalitis following varicella-zoster infection in immunocompetent children has been linked with childhood stroke (Askalan et al., 2001) and cerebral vasculitis, which may present years after initial infection (Hausler et al., 1998).
1.4.2.5 Rubella encephalitis

Rubella is an RNA virus of the Togaviridae family. Encephalitis is a very rare consequence of rubella infection with few irreversible changes and very low incidence of sequelae (Kenny et al, 1965). Most cases are acquired in utero leading to congenital rubella syndrome (CRS) and more rarely post-infectious encephalitis and progressive rubella panencephalitis (PRP) (Townsend et al, 1975; Frey, 1997). Only in CRS has the virus been demonstrated within the brain (Weil et al, 1975; Cremer et al, 1975) suggesting that molecular mimicry may be causing an autoimmune response in PRP.

1.4.2.6 Enterovirus encephalitis

Enteroviruses, being RNA viruses, are able to mutate rapidly and consequently can give rise to new diseases. Enterovirus 71 has been identified in a number of outbreaks with resultant paralysis (Chumakov et al, 1979) and in later Asian epidemics of the late 1990s which resulted in deaths from encephalitis, mainly in children under five (Lum et al, 1998; Huang et al, 1999). Disease was spread by the enteric route and possibly by respiratory infection as well (Ho et al, 1999).

1.4.2.7 Human immunodeficiency virus and encephalitis

Infections with human immunodeficiency virus (HIV) type 1 can often lead to opportunistic infections of the CNS; neurological complications being the primary manifestation of acquired immune deficiency syndrome (AIDS) in 10 to 20% of cases (Mamidi et al, 2002). Toxoplasma gondii is an obligate intracellular protozoan that causes toxoplasmosis, which is generally asymptomatic. However, the loss of cellular immunity in AIDS can allow reactivation of latent infection (Cohen, 1999). Up to 10% of AIDS patients will develop cryptococcal meningitis as a result of their illness. Cryptococcus neoformans is a fungus acquired from the environment, which
develops a capsule in the tissues, but it has not been determined whether it is new infection or reactivation of latent infection when it is associated with AIDS (Mamidi et al, 2002). As with toxoplasmosis, the CD4+ T lymphocyte count will usually be below 100 cells/μl before the infection becomes manifest. CMV infections of the CNS usually occur when the CD4+ count falls below 50 cells/μl and manifest with either dementia (Holland et al, 1994) or ventriculoencephalitis (Kalayjian et al, 1993).

1.4.3 Bacterial meningitis

A number of bacteria are known to cause encephalitis or meningitis. With the rise in HIV infection tuberculosis, and hence tuberculous meningitis, is becoming more prevalent. One study (Porkert et al, 1997) reported a 41% fatality rate in an American hospital. It is difficult to diagnose clinically, often relying on culture, which is slow and can be insensitive, or on histological staining, which is also insensitive (Thwaites et al, 2002). As a consequence both dot blots (Mathai et al, 2003) and ELISA (Sada et al, 1983) have been used for rapid detection of tuberculous meningitis.

Laboratory investigation of tuberculous meningitis shows a raised polymorphonuclear white cell count and reduced glucose in the CSF, possibly along with xanthochromia. Syphilis is another well-known disease with neurological complications, which is closely associated with, and exacerbated by, HIV. Historically, neurosyphilis occurred from between five and twenty-five years after initial infection, (being symptomatic in up to 9% of untreated patients, rising to 12% in patients with neurological illness with latent syphilis). However, according to Carmo et al (2001) neurological symptoms and syphilitic meningitis are most frequent within two years of infection. Laboratory investigation of CSF classically shows a reactive CSF-Venereal Disease Research Laboratory slide test (VDRL), elevated protein and pleocytosis though all may be normal (Tomberlin et al, 1994).
Lyme borreliosis is caused by the spirochete, *Borrelia burgdorferii*, which is spread by tick bites. As with syphilis, approximately 10% of patients develop neuroborreliosis, sometimes after periods of latency. In Europe, but not the USA, where it has not been isolated, *Borrelia garinii* seems to be responsible for a higher incidence of neuroborreliosis due to its greater neurotropism (van Dam *et al*, 1993). It occasionally causes encephalomyelitis with a consequent intrathecal synthesis of immunoglobulin (Oschmann *et al*, 1998).

Whipple’s disease, although commonly intestinal, can both involve or be confined to the CNS (Anderson, 2000). The causative organism is a gram-positive bacillus, *Tropheryma whippelii*, identified in 1992 (Relman *et al*). Although first described in 1907 (Whipple), only 81 cases had been reported by 1996 (Louis *et al*, 1996), reflecting the lack of diagnostic guidelines. Since then, PCR has emerged as the most reliable laboratory test (Ramzan *et al*, 1997).

### 1.4.4 Multiple Sclerosis

MS is an inflammatory demyelinating disease of the CNS, more commonly found in the temperate regions of both northern and southern hemispheres (Rosati, 2001). At present the aetiology of the disease is unknown but it is thought to be mediated by an autoimmune mechanism and may have some genetic susceptibility (Granieri *et al*, 2001).

Infectious causes of MS, often viral, have been postulated (Meinl, 1999) and a number of antiviral IgG responses have been found in the CSF of MS patients, particularly to herpes zoster, measles and rubella (MRZ reaction) and EBV (Cepok *et al*, 2005). However, they cannot be identified as any of the OCBs in the CSF of these patients and their low affinity towards the antigens, compared with high affinity in viral encephalitis, suggests they are part of a secondary, or bystander, reaction (Luxton *et al*, 1995). An
investigation by Monteyne et al (1997) into HSE, using antigen blotting of OCBs, found low-affinity polyspecific reactions in 25% of their MS patients. A number of bacteria have been linked to MS and a minor fraction of OCBs have been shown to be directed against *Escherichia coli*, Enterococcus and *Haemophilus influenzae* (Vartdal et al, 1980). However, if any pathogen is related to MS, a well-studied topic, it remains unidentified (Talbot et al, 2001; Steiner et al, 2001). Molecular mimicry has been suggested as a possible mechanism by which infection may induce autoimmunity (Albert et al, 1999) and several viral and bacterial peptides have been identified that can activate T-cell clones specific for myelin basic protein (MBP) isolated from MS patients (Wucherpfennig and Strominger, 1995). Antibodies to MBP in MS patients were investigated by O'Connor et al (2003) using solution-phase radioimmunoassay (RIA) and ELISA, in this study assays of similar sensitivity. Results from both types of assay suggested that anti-MBP antibodies in the serum and CSF of MS patients were of low affinity and hence were not involved in driving any autoimmune processes. More recently, two bacteria have been proposed as infectious causes, *Chlamydia pneumoniae* (Sriram et al, 1999) and *Acinetobacter calcoaceticus*.

1.4.4.1 *Chlamydia pneumoniae* and multiple sclerosis

*Chlamydia pneumoniae* is an obligate intracellular bacterial pathogen, usually of the respiratory tract, but essentially ubiquitous, disseminating from the respiratory tract within monocytes and macrophages. Although thought to be primarily a human pathogen, Chlamydia pneumoniae bacteria have been linked to sporadic bovine encephalomyelitis, a chronic encephalopathy of cattle (Harshfield, 1970). Since being described as uniquely chlamydial in 1989, *Chlamydia pneumoniae* has also been linked with other chronic conditions such as atherosclerosis and Alzheimer's disease (Swanborg et al, 2003).
A study by Sriram et al (1999) reported the presence of Chlamydophila pneumoniae in the CSF of MS patients by culture and PCR at significantly higher levels than in controls, with the conclusion that Chlamydophila pneumoniae could be the infectious cause of MS. These findings prompted a number of studies, the results of which have been collated by Furrows et al (2003), who used PCR and CSF culture to try and replicate Sriram’s findings. However, they could not find any association between Chlamydophila pneumoniae infection and MS. Sriram’s original study did not investigate the serology of either MS patients or controls, unlike some later studies (Boman et al, 2000; Derfuss et al, 2001). Both of these found over half of their control and MS cohorts to be positive for Chlamydophila pneumoniae whilst PCR on the corresponding CSF samples yielded no positives at all. CSF cultures in Boman’s study were also completely negative unlike Hao et al (2002) who did find one positive in each cohort and more positive PCR results in MS and controls but these were not significant and had a far lower incidence than in serum. Kaufman et al (2002) split CSF samples and sent them to four different laboratories for PCR detection of Chlamydophila pneumoniae. Of the four, one reported 73% of MS and 23% of controls as positive. The other three reported all samples as negative, leading the authors to the conclusion that variation in techniques could account for the different results.

A further study by Vojdani et al (2002) attempted to explain a possible cause of autism as exposure to Chlamydophila pneumoniae generating serum antibodies that cross-reacted with a variety of neuron-specific antigens. Although the authors claimed that autistic children showed higher levels of IgG, IgA and IgM against a variety of neuron-specific proteins, including MBP, they did not demonstrate any specificity for any of the nine neuronal antigens tested; rather patients had apparently higher levels of antibody to all of them. The same effect was shown with the non-neural antigens, butyrophilin from milk, Chlamydophila pneumoniae peptide 483 and streptococcal M protein, possibly
reflecting an inappropriate sample dilution, lack of measurement or correction of serum IgG or any combination of the above. Crucially, it was not possible to obtain CSF samples, even though the antibody effects must have been within the CNS, nor was blood-brain barrier function assessed.

A prospective study (Munger et al, 2004) covering two large cohorts in the USA, of three million USA Army personnel and over 120,000 from a Medical Care Program, measured serum IgG and IgM by microimmunofluorescence (MIF) in samples taken prior to onset of MS. They found 83 and 46 cases of MS respectively, but could not find any link between seropositivity or antibody titres and risk of developing MS, except a slightly increased risk in the smaller cohort. As a result, the authors concluded that although seropositivity and antibody titres could not predict risk of developing MS, they could not say that previous infection with Chlamydia pneumoniae did not alter the risk of developing MS.

1.4.4.2 Acinetobacter and multiple sclerosis

Acinetobacter sp. are commonly found in soil and water and are often part of the commensal flora of man and animals. They are implicated in many nosocomial infections, including secondary meningitis, but are most commonly seen in nosocomial pneumonia in ventilated patients on intensive treatment units (Bergogne-Berezin and Towner, 1996). An amino acid sequence homology was found between the enzyme, 4-carboxymuconolactone decarboxylase (4-CMLD), present in Acinetobacter calcoaceticus 23055 (Ebringer et al, 1997) and a sequence of MBP (amino acid residues 110-124), encephalitogenic in guinea pigs (Eylar et al, 1970). The same sequence was also found in the γ-isoform of the enzyme, γ-CMLD, in Pseudomonas aeruginosa (Hughes et al, 2001). This same study investigated serum antibodies against five strains of Acinetobacter (including Acinetobacter calcoaceticus), Pseudomonas aeruginosa.
and *Escherichia coli* as a control organism on the basis that it contains the amino acid sequence under investigation but in a different enzyme (www.ebi.ac.uk). The authors demonstrated elevated levels of serum IgG, IgM and IgA against all five *Acinetobacter* strains and *Pseudomonas aeruginosa*, but not *Escherichia coli*, in MS patients but not in healthy controls. Raised antibody titres were also found against MBP and neurofilaments in the MS patients but not the controls. Using the absorbance values from the ELISAs for the IgG assays for MBP, *Acinetobacter* and neurofilaments an index was developed to try to distinguish MS from the controls.

Using recombinant peptide Hughes *et al* (2003) showed increased serum IgA, IgM and IgG to a peptide containing the common sequence between *Acinetobacter calcoaceticus* 4-CMLD and MBP. They also found a sequence homology between subunit A of 3-oxoadipate CoA transferase in *Acinetobacter sp.* and myelin oligodendrocyte glycoprotein (MOG) and showed increased levels of serum IgA, IgG and IgM against a recombinant peptide containing the sequence in MS patients. The study was controlled with healthy patients and patients with cerebrovascular accident (CVA).

*Acinetobacter sp.* had previously been proposed as the causative agent of bovine spongiform encephalopathy, (BSE), (Ebringer *et al*, 1997). The proposed mechanism was by molecular mimicry, based on evidence presented later (Hughes *et al*, 2001) as a possible cause of MS. Further work by the same group (Tiwana *et al*, 1999) found serum autoantibodies of IgA type that cross-reacted with sequences in *Acinetobacter calcoaceticus*, bovine neurofilaments and myelin. On this basis they developed an ELISA that used the antibody response to *Acinetobacter calcoaceticus* as an indirect means of detecting BSE in live animals (Wilson *et al*, 2003). The theory was not widely accepted and in 2002 Nielsen *et al* tested a wide variety of animals infected with transmissible spongiform encephalopathies (TSEs) as well as non-infected controls using an assay including both whole cell and lipopolysaccharide from *Acinetobacter sp.*
in an ELISA format. They failed to find significantly raised levels of IgA against

*Acinetobacter calcoaceticus* which, taken with varying normal levels of antibodies to

*Acinetobacter sp.*, resulted in “considerable overlap” between infected and non-infected

populations. Nor could they find significance in measuring antibodies to MBP or

looking for polyreactive antibodies, tested by absorption with *Brucellosis abortis.*
1.5 Introduction to Methods

1.5.1 Isoelectric focusing

Proteins are made up of amino acids that have carboxylic side-groups and amino, imidazole and guanidine side-chains. When the pH is low, carboxylic side-groups are neutral:

\[ \text{R-COO}^- + \text{H}^+ \rightarrow \text{R-COOH} \]

and at high pH they are negative:

\[ \text{R-COOH} + \text{OH}^- \rightarrow \text{R-COO}^- + \text{H}_2\text{O} \]

Amino, imidazole and guanidine side-chains are positive at low pH:

\[ \text{R-NH}_2 + \text{H}^+ \rightarrow \text{R-NH}_3^+ \]

and neutral at high pH:

\[ \text{R-NH}_3^- + \text{OH}^- \rightarrow \text{R-NH}_2 + \text{H}_2\text{O} \]

Glycosylation and phosphorylation will also influence net charge. Thus for any protein molecule there is a pH at which it will have a net charge of zero. This is called the isoelectric point (pI) and is the fundamental principle on which the technique of IEF is based. A mixture of proteins, such as serum or CSF, is electrophoresed on a gel, usually agarose, containing molecules called ampholytes. These are usually synthetic polymers based on repeating amino acids of gradually increasing size, which therefore have a wide range of pIs. When they are subjected to an electric current these ampholytes will move through the gel, the positively charged to the anode and the negatively charged to the cathode. Ampholytes with the lowest pI migrate to the anode and those with the highest pI to the cathode whilst the intermediate ampholytes migrate to the points in between, so determining the pH and hence a smooth pH gradient. Although ampholytes have a high rate of diffusion owing to their small size, they will diffuse away from their pI and return electrophoretically ensuring the
gradient remains smooth. IEF is best run at a constant temperature of approximately 10°C (Westermeier, 2001).

1.5.2 Principle of Eastern Blotting

Eastern blotting (EB) investigates antibodies in a sample using their affinities for antigen. The initial separation of the antibodies in the sample is by IEF. The antibodies are then transferred to a membrane impregnated with the antigen(s) of interest by pressure blotting (Figure 1.4). Antibodies with any affinity towards the antigen(s) are retained on the membrane until it is subsequently incubated with various concentrations of chaotrope. The remaining antibody on the membrane is then detected with a double-antibody system. The resulting patterns can be interpreted visually or quantified using densitometry.

1.5.2.1 History of Eastern Blotting

A variety of methods routinely available in the early 1980s, such as complement fixation, were capable of showing that an immune response had been raised against a particular pathogen but did not have the specificity to show the clonality of the response, i.e. whether it was a polyclonal, oligoclonal or even monoclonal response. The technique of immunoblotting was developed (Moyle et al, 1984) to investigate viral-specific oligoclonal IgG and was largely based on the method of IEF of Walker et al (1983). Moyle et al (1984) used crude viral antigen, from infected cell culture, to impregnate nitrocellulose membrane, which was then laid over a gel containing samples focused by IEF. As the sample proteins were transferred to the membrane by passive blotting, the antibodies with specificity for the viral antigens would recognise and bind
with the antigens in the membrane. These antibodies could then be detected immunologically. This approach meant that all antibodies raised in the subject against the infective agent could potentially be detected in the assay and also negated the need to concentrate CSF prior to electrophoresis which can give rise to artefacts (Hershey and Trotter, 1980).

This original work looked at oligoclonal IgG in the CSF raised against a measles glycoprotein in patients with SSPE, a measles infection of the CNS, and was negatively controlled with CSF from patients with other inflammatory neurological conditions. Moyle and Thompson (1985a) expanded on this study to investigate restriction in the light chain “specificity for antigen” of the specific IgG in SSPE but found that there was no dominant type. They then looked at HSE (Moyle and Thompson, 1985b) using the immunoblotting technique to demonstrate intrathecal synthesis of specific IgG.

Inflammatory diseases of the CNS can impair blood-brain barrier function allowing antibodies to cross into the CSF from the serum. It is therefore important to be able to distinguish intrathecal and systemic humoral immune responses by assaying paired CSF and serum from the patient. The method proved capable of showing that IgG specific for herpes simplex was being synthesised intrathecally and did not come from a serum response.

Thus far, all the work on antigen immunoblotting had been done using nitrocellulose membranes. Nespolo et al (1987) found that polyvinylidifluoride (PVDF) membranes, thicker and stronger than nitrocellulose, gave greatly enhanced sensitivity for protein detection. Although binding lower amounts of protein than nitrocellulose, the higher retention capacity for the protein molecules bound to the membrane surface produced the effect. Luxton and Thompson (1989) explored the use of PVDF in antigen immunoblotting, coating the membrane with a variety of antigens and using random paired serum and CSF samples positive for oligoclonal IgG by IEF. They concurred that
patterns on PVDF were more intense but also found that some samples had different band patterns on the reverse side of the membrane and on a second membrane. Polyclonal background IgG was only found on the obverse side of the first (coated) membrane. They postulated that this phenomenon was due to the differing affinities of the oligoclonal IgG bands for the antigen; the higher the affinity the quicker the antibody binds whilst lower affinity clones will pass into the membrane and onto the reverse side. Alterations in the amount of antigen loaded onto the membrane seemed to confirm this, less antigen resulting in a less intense pattern on the obverse.

Antigen immunoblotting was employed by Mavra et al (1992) as part of their strategy to look at specificity of IgG paraproteins against a range of neurotropic agents. On a qualitative ELISA screening technique thirteen of thirty-four patients with paraprotein bands on IEF were positive for at least one antigen, after immunoblotting only one patient was positive showing that antigen immunoblotting can be a very discriminating test. Monteyne et al (1997) conducted a study comparing detection of intrathecal synthesis of anti-herpes simplex IgG by antigen-mediated immunoblotting and calculation of antibody index following ELISA techniques. Twenty-seven paired samples from fifteen patients diagnosed by CSF PCR were compared to MS patients acting as controls. Both methods detected 14/15 HSE patients, the exception being a patient with relatively benign HSV type 2 encephalitis, which the authors ascribed to either reduced sensitivity of the assays with regard to type 2 HSV, low viral load or false negative.
Stage 1: Antigen covalently binds to membrane

Stage 2: Blocking membrane

Stage 3: Antigen-specific IgG from IEF binds to antigen

Stage 4: Detection of antigen-specific IgG

Key:
- Ultrabind membrane (___) with aldehyde group (|)
- Antigen
- Tris
- Block - milk proteins
- Antibody
- HRP
- HRP-linked antibody
- First antibody

Figure 1.4 Schematic diagram of Eastern blotting.
1.6 Covalent binding membrane

For the work, Ultrabind™ US450 membrane was used, which is a patented (USA patents 4961852 and 4824870) covalent binding affinity membrane (Pall). The membrane is made of modified polyethersulphone and contains aldehyde groups which bind any molecule containing a primary amine (see Figure 1.5). The membrane has a pore size of 0.45 µm, the same as the nitrocellulose membrane routinely used in the laboratory, and an IgG binding capacity of 135 µg/cm², superior to that of nitrocellulose (80 µg/cm²).

![Chemical structure](image)

**Figure 1.5 Binding of antigen to Ultrabind membrane via amide linkage.**

Key: PES, polyethersulphone membrane (Ultrabind); RCOH, aldehyde group on Ultrabind membrane; RNH₂, primary amine group on antigen.

1.7 Western blotting

Western blotting, also known as Western transfer is a technique used for separating proteins in a mixture and then transferring them onto a membrane for any further investigations. The proteins are separated on the basis of their molecular weights by electrophoresis in a polyacrylamide gel (PAGE), and are then electrophoretically blotted from the gel onto the membrane.
1.8 Quantification of bands by densitometry

Densitometry is a form of photometry used to measure electrophoresis lanes. It can be used to measure transmittance or reflectance of light, for clear and opaque media respectively, to produce a densitogram, also called a peak diagram. The area(s) under the peak(s) can then be quantified using a variety of software programs available. Densitometric scanning is most commonly used to quantify serum protein fractions after electrophoresis; the percentage values from the densitometry being used to calculate actual values from total protein estimation. It can also be applied to IEF patterns (Paluch et al, 1984).

1.9 Total protein estimation

The assay used to measure total protein was the Bio-Rad DC Protein Assay (Bio-Rad), which detects protein colorimetrically following solubilisation in detergent. The assay consists of two stages; firstly, protein reacts with copper in an alkaline medium (tartrate), secondly, the copper-treated protein reduces Folin reagent by removing one, two or three oxygen atoms. This results in reduced species of a blue colour, the absorbances of which can then be read and used to calculate concentration.

1.10 Rocket electrophoresis

Samples are added to cylindrical wells cut into an electrophoresis gel containing antibodies to the protein to be measured. When electrophoresed the sample protein of interest will migrate through the gel and react with the antiserum, forming an insoluble complex, which therefore precipitates. As the serum antigen is depleted the line of precipitation tapers to a point and the length of the resulting “rocket” is proportional to the concentration of the antigen in the sample. The presence of polyethylene glycol
(PEG) promotes precipitation of the immune complex by competing for water of solvation with the immune complex.
2.0 Methods

2.1 Isoelectric focusing

2.1.1 Isoelectric focusing gel manufacture

A 22.6 cm x 11.5 cm gel was made by adding 0.3 g of IEF agarose (Amersham Pharmacia) to 30 ml of deionised water in a glass bottle with a screw cap. The cap was lightly screwed on because the bottle was then placed in a boiling water bath until the agarose was fully dissolved. Some methods include 3.6 g sorbitol and 3 ml glycerol in the gel mixture but these are not absolutely necessary. Both were originally included in gel recipes to minimise diffusion of bands when the gel itself was stained for interpretation. Thus, the gels were made using just 30 ml of deionised water with 0.3 g IEF agarose. The molten gel was then transferred, still in the bottle, to a water bath at 65 °C and given at least one hour to equilibrate to the lower temperature.

Whilst the gel was cooling a casting frame was constructed. A sheet of GelBond (Serva) was cut to 25 cm x 13.5 cm, the same dimensions as a glass plate, 3 mm thick. A small pool of 50% methanol (Hayman): 50% water mixture was squirted into the centre of the glass plate and the GelBond fitted squarely on it. Both sides of the GelBond are hydrophilic.

Once the GelBond was on the glass plate, a length of paper towel was placed across the top and the excess methanol: water squeezed out using a photographic print roller. A plastic template (Amersham Pharmacia) was laid on the GelBond, smooth side down to ensure best contact, and the whole apparatus secured using bulldog clips. Once it was determined, by spirit level, that the gel casting apparatus was level, it was heated using a hairdryer.
Two ml of pH 3-10 and 0.5 ml pH 8-10.5 ampholyte solutions (Amersham Pharmacia) were added to the molten gel, using a syringe (Becton Dickinson). The ampholytes were mixed into the molten gel by swirling the bottle, after which the bottle was recapped, and returned to the water bath briefly to regain temperature. When the plate was warmed, the hairdryer was switched off and the bottle of molten gel was taken from the water bath. The gel was poured carefully into the casting frame where the neck of the bottle was used to ensure the gel was evenly spread within the whole frame and that any air bubbles were moved to the edges of the frame. The gel was allowed to cool and set before removal of the bulldog clips and transfer of the gel in the frame to a plastic, lidded box containing a damp paper towel. The gel could then be stored in the damp box at 4 °C for up to a week before use.

2.7.2 Running the gel

The prepared gel (see section 2.1.1) was taken out of the cold room, still in the damp box, and allowed to equilibrate to ambient temperature. The electrophoresis tank (Amersham Pharmacia) on which the blot was run was coupled to a circulating water cooler (Amersham Pharmacia) set at 12 °C. It was necessary to start the cooler at least 30 minutes before the scheduled beginning of the run to ensure that the plate of the tank was at a constant temperature.

Whilst the gel was equilibrating the wicks were put in to soak. These were strips of blotting paper (Hollingsworth and Vose), cut to slightly less than the length of the gel (22 cm) and approximately 6 mm wide. Three of these, laid on top of each other, formed one wick. Two wicks were soaked, one in 0.05M sulphuric acid and the other in 1M sodium hydroxide.

The gel was taken from the damp box, lifted away from the glass plate and carefully separated from the casting frame. Approximately 1 ml of 50% methanol: 50% water
mixture was squirted onto the centre of the cooling plate and the gel, on the GelBond, was laid squarely onto the cooling plate, the methanol: water providing an airtight seal between the GelBond and the cooling plate. Any excess methanol: water leaking out from the edges of the gel and onto the GelBond was dried away with a tissue. Using a scalpel blade the four sides of the gel were trimmed by approximately 2 mm. This removed the slightly raised edges, which result from the casting, best described as a “meniscus” effect, which may potentially affect the contact of the wicks and “soakaways”, to be mentioned later. Once the gel was trimmed it was blotted three times with nitrocellulose membrane, cut to 20 cm x 10 cm, to remove excessive moisture from the surface of the gel. The membranes were left on the gel until they were soaked through and were then removed. Next, an application foil (Amersham Pharmacia, discontinued) was laid on the gel, approximately 3 cm up from the bottom edge of the gel, i.e. the edge nearest the experimenter, and central with regard to the vertical edges of the gel.

The application foil was then pressed gently but firmly onto the gel to ensure that it made an airtight seal. If the seal is not airtight, when the samples are applied they may be drawn away from the well by capillary action resulting in smeared and indistinct patterns that are difficult to interpret.

Samples were then applied, from right to left, leaving the outside wells either unused or containing distilled water. When affinity studies are being done that require the membrane to be divided after blotting, marks can be made with waterproof ink on the upper and lower edges of the GelBond, which lay outside the cast gel, corresponding to where the division will be.

The wicks were first blotted in paper tissue and then placed on the gel; the sulphuric acid-soaked wick at the anode and the sodium hydroxide-soaked wick at the cathode. The anodal wick should not touch the application foil and the cathodal wick should be
placed at least 1 cm in from the top of the gel. Once the wicks were in place paper
towels were placed at the anodal and cathodal edges of the gel, though not touching the
wicks, to absorb the moisture generated as the gel was run.
The electrodes on the glass plate were adjusted so that the wire ran along the centre of
the wick. The electrodes were secured in position using their integral screw clamps and
the plate placed in position using the feet at the corners fitted into the appropriate holes
in the tank. The leads from the electrodes were connected and the lid of the apparatus
put on. The lid was connected to the power supply (EPS 3501, Amersham) and the gel
is run at 900 volts, maximum 150 watts and 20 mA for 900 volt hours.
During the early part of the run, somewhere between 200 and 400 volt hours, runs were
paused to allow the soakaways to be replaced, the application foil to be removed and
cleaned and excess moisture to be blotted from the wicks. The electrode plate was then
replaced and reconnected and the run continued until completion.

2.1.3 Blotting the gel

Once the gel had finished running the electrophoresis tank lid and the electrodes were
removed to expose the gel. Firstly, the soakaways were removed, and then the wicks
were taken off, all being discarded. A piece of nitrocellulose (BioRad), approximately
10 cm x 20 cm, was laid on the gel for about thirty seconds to remove any moisture and
protein lying on the surface of the gel. The membrane impregnated with the protein(s)
of interest was then carefully laid on the gel ensuring that there was no air trapped
underneath it. If air does become trapped then the bubbles can be squeezed out using a
glass rod rolled gently but firmly from the centre of the membrane to the edge.
Once the membrane was in place six pieces of blotting paper were placed on top
followed by a glass plate and a 1 kg weight. This was left in place for ten minutes. The
apparatus was then dismantled and the membrane taken for further experimentation.
2.1.4 Incubating with sodium thiocyanate solutions

For affinity studies using NaSCN (Sigma) the membrane was left on the gel after the weight, glass plate and filter paper had been taken off. Whilst still in situ the blotted membrane was cut, using a surgical blade and true-edged ruler, using the marks in waterproof ink made on the edge of the GelBond when the samples were applied (see Section 2.1.2). The pieces of membrane were then labelled appropriately and incubated with NaSCN solution in a tray, 10 cm x 10 cm, (Elkay) for the required length of time. Once the incubation was finished the membranes were washed in twenty rapid changes of running tap water to remove any NaSCN from the experiment prior to being developed.

2.1.5 Development of the membrane

Membranes that had been incubated with NaSCN were reincubated in blocking solution (see section 2.2.1) for at least thirty minutes to ensure that any blocking protein that may have been stripped by incubation with NaSCN was replaced, prior to incubation with the first antibody.

The membranes, measuring approximately 10 cm by 5 cm, were incubated in first antibody, appropriately diluted, and were incubated overnight on a slow rocker (Stuart Scientific) at 4 °C. The following morning the membranes were brought back to room temperature on a slow rocker before the antibody solution was decanted off. Each membrane was then washed in five changes of 25 ml of 0.025% Tween 20 (Sigma) in 0.2% skimmed milk/0.9% saline, changed every ten minutes. Membranes were then incubated in secondary antibody, appropriately diluted, for two hours at room temperature on a slow rocker. The secondary antibody was decanted off and the membranes washed in distilled water. The membranes were then kept in saline until the colour reagent was ready.
2.2 Coating membranes

2.2.1 Coating the membrane with human serum albumin

A solution of human serum albumin (HSA) (Sigma) was made by dissolving 2.5 g of HSA in 100 ml of deionised water to give a stock solution of final concentration of 25 g/l.

Ultrabind US450 (Gelman) membranes, cut to 10 cm x 20 cm on a guillotine, were placed in a dry polypropylene incubation chamber and soaked through with 25 ml of 0.9% saline. 400 µl of the stock solution of HSA was mixed into a further 25 ml of saline and added to the membrane in the incubation chamber. Thus, the final amount of HSA in the incubation chamber was 1000 µg, or 1 mg. The membrane, 200 square centimetres, is theoretically coated with 5 µg of HSA per square centimetre. Ultrabind 450 has a quoted total binding capacity of 135 µg per square centimetre; thus it is bound at 3.7% of total binding capacity.

Once the membrane was incubating in the antigen solution, the lid was placed on the incubation chamber and the whole was transferred to a slow rocker at 4 °C overnight. The following morning the incubation chamber and its contents were allowed to return to ambient temperature, on a slow rocker, before the antigen solution was decanted. The membrane, still in the incubation chamber and on the rocker, was washed twice for ten minutes in tap water. This removed weakly bound protein from the membrane. Once the tap water had been decanted the membrane was blocked. The blocking solution was 2% milk in saline i.e. 2 g of dried skimmed milk powder (Marvel) dissolved in 100 ml of saline with 1% Tris (Sigma).

The membrane was blocked for at least one hour, although it could have been left longer, before the blocking solution was decanted and the membrane washed twice in cold tap water. The membrane was transferred, using clean forceps, onto a piece of
blotting paper where it was allowed to air dry before being put between two clean
pieces of blotting paper and stored in a sealed plastic bag at -20 °C. Membranes kept
this way have been stored for up to a week before use with no discernible deterioration
in the final blot.

2.2.2 Coating a membrane with bacterial and viral proteins

2.2.2.1 Preparation of bacterial cultures

The primary culture of *Acinetobacter calcoaceticus* (NCIMB 16904) was obtained from
the National Collections of Industrial and Murine Bacteria Ltd. (Aberdeen, Scotland).
The Department of Microbiology at King’s College, London provided primary cultures
for *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Escherichia coli*.
The cultures were grown in one litre flasks on an orbital shaker for two days at 30 °C
for *Acinetobacter calcoaceticus* and at 37 °C for *Pseudomonas aeruginosa*, *Proteus
mirabilis* and *Escherichia coli* in 200 ml nutrient broth (Oxoid: 25 g/l). Flasks were
inoculated with two loopfuls of starter culture and left shaking for 6 hours at 37 °C.
Batch culture cells were harvested by centrifugation at 4000 rpm for 20 minutes at less
than 10 °C (Beckman JA-20 rotor 6 x 250ml). Pellets of cells were completely
resuspended in BugBuster (Novagen) (3 ml/50 ml nutrient broth) and incubated on a
shaking platform for 20 minutes at room temperature.
The lysed bacterial culture was diluted 3:1 with 80 mg/ml sodium dodecyl sulphate
(SDS) (BDH) solution and 0.5M dithiothreitol (DTT aka Clelands reagent) (Sigma)
added at 1/20th of the original volume of culture and SDS. The container was then
sealed and placed in a 65 °C bath for fifteen minutes.
Prior to use the total protein of the bacterial preparation was determined using a
modified Lowry method (Lowry *et al*, 1951) (see Section 2.6).
2.2.2.2 Coating the membrane with bacterial antigens

Ultrabind US450 membranes (Gelman), cut to 10 cm x 20 cm, were placed in an incubation chamber and allowed to soak thoroughly in 25 ml of 1mM ethylenediaminetetra-acetic acid (EDTA) (BDH)/0.9% saline. The processed bacterial antigen was mixed into a further 25 ml of 1mM EDTA/0.9% saline and added to the incubation chamber at sufficient volume to coat the membrane at 5 \( \mu g \) per square centimetre. The sealed chamber was incubated overnight at 4 \(^\circ\)C on a slow rocker. After returning to room temperature the antigen solution was decanted off and the membrane washed in two changes of tap water. Available protein binding sites were blocked with 1mM EDTA/2% w/v skimmed milk/0.1% Tween/0.9% saline for two hours. Once blocked, the membrane was rinsed in tap water and left to air-dry between two sheets of blotting paper before being placed in fresh blotting paper and stored in a sealed plastic bag at –20 \(^\circ\)C. Membranes could be stored for up to one week with no visible deterioration on the final blot.

2.2.2.3 Coating the membrane with commercial virus preparations

Measles and Herpes antigens: the antigen was supplied freeze-dried (Quadratech) and reconstituted in 1 ml of ultrapure distilled water. Total protein was estimated using the Lowry method. Membranes were coated at 50 \( \mu g \) total protein per square centimetre of membrane by mixing the appropriate quantity of reconstituted antigen into 25 ml of saline. This was immediately added to a membrane already soaking in 25 ml saline in an incubation chamber, which was then sealed and incubated overnight on a slow rocker at 4 \(^\circ\)C. The following morning the membrane was briefly rinsed in tap water and blocked in 2% w/v skimmed milk/1% w/v Tris in 0.9% saline for at least one hour. After blocking, the membrane was rinsed in tap water and left to air dry before being stored as above (see Section 2.2.2.2).
2.3 Western blotting for *Chlamydophila pneumoniae*

Lithium dodecyl sulphate (LDS) (Invitrogen) sample buffer was used rather than the sodium dodecyl sulphate (SDS), as LDS remains soluble for longer thus enabling antigen preparations to be stored for longer. Also, denaturation of proteins with LDS can be successfully done at 65 °C as opposed to 100 °C with SDS, which thus prevents some loss of labile proteins. DTT (Invitrogen) was also added to prevent the formation of disulphide bonds between cysteine residues.

2.3.1 Preparation of *Chlamydophila* antigen for Western Blotting

The cell pellets, control and infected, were washed. 1 ml of saline was added to the pellets and the bottle gently shaken by hand to dislodge the cells. They were then centrifuged at 4000 rpm and the supernatant removed. This was repeated twice more. Pellets were then suspended in 300 μl each of T-Per (Pierce) and BugBuster (Novagen) and incubated at 56 °C for 25 minutes before being frozen at −80 °C.

2.3.3 Western blot

The desired dilution of antigen preparation was determined by serially diluting the antigen preparation (1/2, 1/4, 1/8, 1/16 and 1/32) in dilute LDS solution (1 part LDS to 3 parts deionised water). 25 μl of each dilution was electrophoresed on a NuPage 4-12% Bis/Tris 10 well precast gel (Invitrogen) at 200 V, 120 mA and 25 W for 35 minutes in a buffer tank with electrode and power pack (Invitrogen) in 2-[N-Morpholino]ethanesulphonic acid (MES) running buffer (Invitrogen). The gel was transferred to fixative solution and was then stained with Coomassie Brilliant Blue (Invitrogen) to visualise the protein bands present for assessment of optimum dilution in the subsequent Western blotting.
Cell line homogenates were prepared by adding 40 µl of homogenate, 50 µl of LDS, 20 µl of 0.5M DTT (to a final concentration of 0.05M) and 90 µl of deionised water, thus giving a final dilution of approximately 1 in 5 for the antigen preparation and 1 in 4 for the LDS. The preparation was then incubated at 65 °C in a preheated water bath (Grant) for 15 minutes in a snap-top vial (Eppendorf). Homogenates were cooled at ambient temperature and mixed on a vortex mixer (Stuart Scientific).

The 220 µl of antigen preparation was applied to the large well of a NuPage 4-12% Bis/Tris 2D gel (Invitrogen) with 3 µl of SeeBlue Plus molecular weight marker (Invitrogen) in the small well and run as for the serial dilutions (above). The gel was electroblotted to transfer the proteins onto a Biodyne membrane (Gelman) for subsequent investigations. Western transfer was carried out using a semi-dry electroblotting system (Invitrogen) with transfer buffer (Invitrogen).

The electrophoresed gel was removed from its plastic cassette by breaking off the back of the cassette and applying a piece of filter paper, 9 cm², soaked in transfer buffer. The gel was then eased away from the front piece of the cassette by adhering to the filter paper. This was then placed into the bottom of the blotting cassette, containing the cathode electrode, which contained two buffer reservoirs, 9 cm², and three pieces of filter paper, all soaked in transfer buffer. A piece of Biodyne, 9 cm x 9 cm, soaked in transfer buffer, was then laid onto the gel; any air bubbles between them were gently squeezed out to the edge of the membrane. Two more pieces of soaked filter paper were laid on top, followed by a piece of porous cellophane (Amersham Pharmacia), 9 cm square, soaked in transfer buffer, four more pieces of soaked filter paper and two more buffer reservoirs. This “sandwich” was then repeated, thus allowing two gels to be blotted simultaneously. The top of the blotting cassette, containing the anode, was put on the top and the complete cassette inserted into the electrophoresis tank. The electrophoresis tank was filled with tap water to surround the blotting cassette and
provide a medium for heat exchange to remove the heat generated by the transfer process. Electroblotting was for one hour at 25 V, 160 mA and 17 W at room temperature.

Once blotted, the membrane was removed from the blotting cassette and placed into a blocking solution of 2% w/v skimmed milk powder in 0.9% saline on a slow rocker for at least one hour. This was to block any unbound protein binding sites with milk protein and hence reduce background when the membrane was finally developed and visualised. The blocking solution was rinsed off with two changes of 0.9% saline. Membranes were then either dried at room temperature between two pieces of filter paper and stored at -20 °C in a sealed plastic bag, or used immediately. Membranes stored were always used within one week of preparation without any noticeable deterioration in quality when compared to fresh membranes.

2.3.4 Probing of membrane

The blotted and blocked membrane was then placed into a ten-lane incubation manifold (Hoefer) and 2 ml of the diluted serum samples were applied. Serum samples were diluted 1:100 in 0.2% w/v skimmed milk in 0.9% saline by adding 50 µl of vortex mixed serum to 5 ml of the diluent. The loaded manifold was left to incubate overnight at 4 °C on a slow rocker. The following morning the manifold was allowed to equilibrate to room temperature on a slow rocker before being washed. 2 ml of wash solution (0.9% saline/0.2% w/v skimmed milk/0.05% Tween 20) was applied to each lane for ten minutes on the slow rocker. This was repeated ten times. The membrane was then removed from the manifold and incubated with 25 ml of rabbit anti-human IgG, IgM, IgA, κ light chain and λ light chain conjugated to HRP (Dako), diluted 1/1000 in 0.9% saline/0.2% w/v skimmed milk for one hour at room temperature on a slow rocker. The membrane was then washed ten times in 25 ml of wash solution before
being transferred into saline prior to visualisation with enhanced chemiluminescence (ECL).

2.3.4.1 Enhanced chemiluminescence

This procedure was performed in its entirety in a darkroom under safelight. The membrane was taken out of saline and placed on a sheet of blotting paper to remove excess saline. 5 ml each of SuperSignal® West Pico Luminol enhancer solution and SuperSignal® West Pico stable peroxide solution (both Pierce) were mixed in a plastic boat, 10 cm by 10 cm. The membrane was immersed in the luminol/peroxide solution for five minutes with occasional agitation by hand to prevent product accumulation. The membrane was then taken out of the luminol/peroxide solution and placed on blotting paper to remove the excess luminol/peroxide solution before it was sandwiched between two sheets of clear acetate. The “sandwich” was constructed in a Hypercassette™ (Amersham), a light-proof lidded box. Under safelight a sheet of chemiluminescence film (Hyperfilm™, Amersham Pharmacia) was placed over the blot(s) and the lid of the box closed and sealed. After exposure, the film was removed from the Hypercassette™ and developed automatically in an X-OMAT Multiloader 300 (Kodak).

2.4 Other methods used in Chlamydophila pneumoniae in MS study

The author did not perform the assays detailed in sections 2.4.1, 2.4.1.1, 2.4.2 and 2.4.3.

2.4.1 Culture of Chlamydophila pneumoniae from cerebrospinal fluid

The culture method was based on that used by Sriram et al (1999), with some modifications. HL and Hep2 cell lines were used for culture (Kuo and Grayston, 1990; Roblin et al, 1992). Approximately 400 µl of CSF was collected directly into 1.5 ml
microcentrifuge tubes. Trypsin 0.25%/EDTA 1mM (Gibco BRL) was added to a final concentration of 0.1% trypsin. The sample was vortex mixed, incubated at 35 °C for 30 minutes, then mixed again and centrifuged at 13,000 rpm for 45 minutes. The supernatant was discarded and the pellet resuspended in 1 ml Eagles growth medium (GibcoBRL) with 10% fetal calf serum (Globepharm) and added bicarbonate, glutamine and vitamins. The resuspended sample was divided between 2 HL and 2 Hep2 monolayers. The monolayers had been prepared in advance on coverslips, washed with Dulbecco’s buffered saline (MP Biomedicals) 4 times, treated with DEAE-dextran 30 mg/ml (GibcoBRL) for 30 minutes and washed 4 more times with Dulbecco’s buffered saline. After the CSF sample was added, the monolayers were centrifuged at 3600 rpm at 4 °C for 1 hour. 1 ml of medium was added to the vials (as before, but with added glucose 0.03M, amphotericin 2.5 μg/ml, vancomycin 10 μg/ml, gentamicin 10 μg/ml and cycloheximide 4 μg/ml). The vials were incubated at 35 °C for 7 days with additional centrifugation on days 4, 5 and 6. On day 7, one monolayer of each type was kept for examination. The other monolayer was passaged by manually scraping off the cells and resuspending them in their growth medium; the cell suspension was divided between 2 freshly prepared monolayers and set up as previously described. These monolayers were incubated for a further 7 days, with centrifugation on days 11, 12 and 13, and were examined on day 14. Monolayers were fixed with methanol and then stained using the IMAGEN chlamydial lipopolysaccharide direct immunofluorescent antibody test kit (DAKO Diagnostics). The stained coverslips were examined under an ultraviolet (UV) microscope by a single, blinded reader.

2.4.1.1 Isolate identification

The cell culture isolate was identified by specific polymerase chain reaction-enzyme linked oligonucleotide assay (PCR-ELONA), polymerase chain reaction-restriction
fragment length polymorphism (PCR-RFLP) and DNA sequencing of the *omp2* gene according to the method of Hartley *et al* (2001).

2.4.2 *Chlamydophila pneumoniae* polymerase chain reaction

CSF aliquots of approximately 400 µl were collected and *Chlamydophila pneumoniae* concentrated by the same method as used for culture. Pellets were frozen at -70 °C and extracted in batches of 10. DNA was prepared from the pellet by a method adapted from Sriram *et al* (1999). For each extraction run, stock lysis buffer was prepared as previously (Sriram *et al*, 1999) but with the addition of approximately 50 elementary bodies of *Chlamydophila trachomatis* (in 0.5 µl of stock suspension 105 EBs/µl (Hartley *et al*, 2001)) *per* patient or negative or positive control sample. *Chlamydophila trachomatis* internal control was omitted from one extraction run. Lysis buffer was added to individual samples, which were vortexed and incubated overnight at 37 °C. Samples were then boiled for 10 minutes and made up to 550 µl with 10mM EDTA pH 7.5; DNA was then extracted by phenol/ chloroform/isoamyl alcohol using the method of Sriram *et al* (1999). This was modified by the addition of glycogen in the final DNA precipitation step. The final DNA pellet was dried at 55 °C for 30 minutes before suspension in 50 µl of DNAase free water (Baxter).

PCR detection followed the method of Hartley *et al* (2001). Briefly, a single round PCR was performed using primers for conserved regions of the outer membrane 2 protein gene present in *Chlamydophila trachomatis* and *Chlamydophila pneumoniae* amplifying a 557 base pair (bp) product from *Chlamydophila trachomatis* and 584 bp product from *Chlamydophila pneumoniae*).

All reactions were carried out in a Perkin Elmer type 480 thermal cycler, in 50 µl reaction volumes. Reactions contained 20 µl of sample, 200µM each dNTP.
(Amersham-Pharmacia), 5 pmol each primer (Ch1 and Ch2-bio) and 1.25 units Taq
gold polymerase (Roche Biochemicals). Amplification conditions were: 95 °C for 10
min, 1 cycle; 94 °C, 1 min., 55 °C 1 min., 72 °C, 1 min., 40 cycles; and a final extension
step of 72 °C, 7 min., 1 cycle.
The species-specific products are individually detected by a sensitive and specific
chemiluminescent ELONA (Hartley et al, 2001). The ELONA is performed in duplicate
for each probe. Under ideal conditions the PCR-ELONA is able to detect a single copy
gene input into the PCR (Hartley et al, 2001). Presence of approximately 50 genome
copies of one species does not prevent detection of a low number of another
(unpublished data, J. Hartley). When the internal control is detected, a negative PCR-
ELONA for Chlamydophila pneumoniae equates to below 50 genomes/400μl of CSF
trypsin-EDTA pellet (125 genomes/ml CSF). Failure to detect the Chlamydophila
trachomatis internal control demonstrates reduced efficiency in the overall process of
extraction, amplification or detection, in that specific sample. As long as routine
external PCR and ELONA positive controls are valid, a negative Chlamydophila
pneumoniae result may be given but the level of sensitivity is not known. However, this
is the usual situation for most assays, as they have no internal control.

2.4.3 Chlamydophila pneumoniae serology

Serum IgM and IgG antibodies to the family Chlamydiaceae, were assayed using
ELISA kits from MEDAC Diagnostika (Hamburg, Germany). The assay was performed
according to manufacturer’s instructions, and included positive, negative and blank
controls.

Any serum samples that were IgM or IgG antibody positive were further tested using
microimmunofluorescence (MIF). MIF, regarded as the gold standard, was devised in
1970 as an assay for Chlamydophila trachomatis (Kuo et al, 1995; Wang and Grayston,
1970) and has been modified to incorporate testing for *Chlamydia pneumoniae*. It is a specific and sensitive assay for chlamydial infection. Serum samples are tested against pools of antigens associated with specific serotypes, including a pool that is specific for *Chlamydia pneumoniae*. The antigens used are derived from the major outer membrane protein. MIF slides were made up in-house in advance by dotting antigen suspensions on to 12-well PTFE coated slides and allowing to air dry. For the assay, dilutions of the patients' sera from 1:8 to 1:512 were made and 10 μl of each dilution placed on each well. The slide was incubated in a humid chamber for 1 hour at 35 °C. Excess serum was rinsed away with PBS and the slide was air-dried. 10 μl of fluorescent conjugate containing anti-human IgG, IgM or IgA (Sigma) was added to the relevant wells and incubated in a humid chamber for 1 hour at 35 °C. The slide was washed, air dried and examined under the UV microscope at high-power oil immersion. A positive result for IgG was taken as a titre of equal to or greater than 256, and for IgM/IgA was a titre equal to or greater than 64.

2.5 Calculation of peak areas

The immunoblot was scanned on a GS-690 scanning densitometer (BioRad) at a resolution of 100 dpi and saved as a proprietary file, `<filename.IMG>`, using Molecular Analyst/PC software provided with the densitometer. This was then converted into a tag image file format (TIFF) file using the same software. The saved image was opened in Irfanview 32 Bit software version 3.51 (Vienna University of Technology, Austria) where it was converted to Greyscale and saved as a non-compressed TIFF file. The image was then opened in ImageJ version 1.31 (Rasband, 1997-2005), a Java-based programme. The image is calibrated using a scan of a Kodak photographic step tablet scanned with an Epson Expression 1680 professional scanner (step-tablet-epson-8bit.tif), available via the National Institutes of Health (NIH) website.
(http://rsb.info.nih.gov/ij/docs/examples/calibration) as a ZIP compressed TIFF file, using the Rodbard curve fitting procedure developed for use with ImageJ (Figure 2.1). Rodbard is a four parameter general curve fit function proposed by David Rodbard at NIH. A box was drawn on the first lane, using the box-drawing tool, and copied to all subsequent lanes. On the command, Plot Lanes, a series of densitograms was generated corresponding to the separate traces on the blot. Using the line-drawing tool the peak area(s) to be measured were marked and then using the tracing tool the pixel densities of the areas were calculated by the programme for both the peaks and the total area under the curve. Peak areas were then expressed as a percentage of the total area (in pixel density) under the curve.

![Figure 2.1 Calibration curve for ImageJ.](http://rsb.info.nih.gov/ij/docs/examples/calibration)

Calculated using Rodbard curve-fitting procedure on data from scanned Kodak step tablet.

2.6 Total Protein Assay (BioRad)

10 µl of standards (0, 0.125, 0.25, 0.5, 1.0 and 2 mg/ml), quality controls (low and high) and unknowns were pipetted, in duplicate, into a standard 96 well ELISA plate. 25 µl of reagent A (alkaline copper tartate) was then dispensed into each well followed by 200
μl of reagent B (Folin reagent). The plate was incubated on the bench for at least fifteen minutes to allow the colour to develop before absorbances were read at 750 nm in a plate reader (Wallac). A standard curve was constructed and the concentrations of the unknowns calculated from the standard curve.

2.7 Samples

2.7.1 Multiple Sclerosis and Chlamydophila pneumoniae study

A total of 48 subjects were recruited for the study of which 46 were analysed in this study. Two control patients were excluded due to lack of sample material. Nineteen subjects, (mean age 39.3 +/- 12.7, 10/19 (52.6%) male), had a clinical diagnosis of MS. Twenty-seven patients, (mean age 49.2 +/- 15.5, 16/27 (59.3%) male), with other neurological disease made up the control group of inflammatory diseases such as encephalitis or neurosarcoidosis, and non-inflammatory diseases such as dementia or epilepsy. Demographic data for each group is given in Tables 2.1 and 2.2. All patients were undergoing routine lumbar punctures for diagnostic purposes. Ethical approval was obtained prior to the study. All samples were coded so that the laboratory work was carried out blinded.

All blood samples were centrifuged at 2800 rpm for twenty minutes and the serum aliquotted into two or three tubes containing approximately 800 to 1000 μl each and labelled with study number.

2 ml of plasma from CPT tubes was aliquotted into two tubes, labelled and frozen, the remainder being mixed with 1.5 ml of phosphate buffered saline, the cells being resuspended and transferred to a V-bottom tube (Sarstedt), labelled PBMC. The tubes were centrifuged at 10000 rpm for 2 minutes, the supernatant removed and all tubes frozen at -70 °C.
Four 800 µl aliquots of CSF were taken; one was divided into two equal aliquots and frozen along with one of the 800 µl aliquots at -70 °C.

Table 2.1 Control patient demographics for *C. pneumoniae* in MS study.

<table>
<thead>
<tr>
<th>Study number</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
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<td>42</td>
<td>M</td>
<td>Optic neuropathy</td>
</tr>
<tr>
<td>4</td>
<td>71</td>
<td>F</td>
<td>Corticobasal degeneration</td>
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<td>5</td>
<td>50</td>
<td>F</td>
<td>Headache</td>
</tr>
<tr>
<td>6</td>
<td>49</td>
<td>M</td>
<td>Whipples disease</td>
</tr>
<tr>
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<td>35</td>
<td>F</td>
<td>Functional disorder</td>
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<tr>
<td>14</td>
<td>41</td>
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<td>Stroke</td>
</tr>
<tr>
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<td>M</td>
<td>Guillane-Barre Syndrome</td>
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<td>Trigeminal neuropathy</td>
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<td>F</td>
<td>Facial nerve disorder</td>
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<td>M</td>
<td>Brain abscess</td>
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<tr>
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<td>69</td>
<td>F</td>
<td>Alcohol-related ataxia</td>
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<td>27</td>
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<td>M</td>
<td>Primary lateral sclerosis</td>
</tr>
<tr>
<td>28</td>
<td>61</td>
<td>M</td>
<td>Cerebral infarct</td>
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<td>30</td>
<td>53</td>
<td>M</td>
<td>Whipples disease</td>
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<td>M</td>
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<td>M</td>
<td>Myelopathy</td>
</tr>
<tr>
<td>38</td>
<td>60</td>
<td>M</td>
<td>Axonal/demyelinating neuropathy</td>
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<tr>
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<td>M</td>
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<tr>
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<td>F</td>
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<td>47</td>
<td>F</td>
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<td>68</td>
<td>68</td>
<td>F</td>
<td>Myelopathy</td>
</tr>
<tr>
<td>69</td>
<td>57</td>
<td>M</td>
<td>Cerebellar ataxia</td>
</tr>
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Table 2.2 MS patient demographics for *C. pneumoniae* in MS study.

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<tr>
<th>Study number</th>
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</thead>
<tbody>
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<td>F</td>
<td>Primary progressive MS</td>
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<td>9</td>
<td>21</td>
<td>M</td>
<td>MS</td>
</tr>
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<td>12</td>
<td>36</td>
<td>F</td>
<td>Relapsing remitting MS</td>
</tr>
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<td>F</td>
<td>Relapsing remitting MS</td>
</tr>
<tr>
<td>15</td>
<td>50</td>
<td>M</td>
<td>MS</td>
</tr>
<tr>
<td>19</td>
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<td>M</td>
<td>Relapsing remitting MS</td>
</tr>
<tr>
<td>25</td>
<td>55</td>
<td>M</td>
<td>Relapsing remitting MS</td>
</tr>
<tr>
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<td>53</td>
<td>M</td>
<td>Relapsing remitting MS</td>
</tr>
<tr>
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<td>21</td>
<td>F</td>
<td>Relapsing remitting MS</td>
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<td>M</td>
<td>Relapsing remitting MS</td>
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<td>F</td>
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</tr>
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<td>F</td>
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<td>MS</td>
</tr>
<tr>
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<td>MS</td>
</tr>
<tr>
<td>63</td>
<td>16</td>
<td>F</td>
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<td>M</td>
<td>MS</td>
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<tr>
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</tr>
<tr>
<td>70</td>
<td>29</td>
<td>M</td>
<td>Relapsing remitting MS</td>
</tr>
</tbody>
</table>

2.7.2 *Multiple Sclerosis and Acinetobacter calcoaceticus* study

**Serum and CSF samples.** Matched serum and CSF samples were obtained from routine patient admissions at the National Hospital for Neurology and Neurosurgery (NHNN), London, United Kingdom. All samples were received in the Department of Neuroimmunology for IEF for OCBs of IgG.

Twelve patients with confirmed MS (5 male, 7 female; age 45 +/- 9.8, World Health Organisation (WHO) International Classification of Diseases (ICD)-10 code G.35) (Table 2.4), and eight other patients with neurological disease; (3 female, 5 male; age 54.3 +/- 14.4) (Table 2.3), were selected for the study. Eleven of the MS patients were recently diagnosed and not on therapy, the twelfth was on β-IFN, having been diagnosed five years previously. Nine of the MS patients had relapsing-remitting MS and three primary progressive MS. Ten controls and 10 MS patients were originally selected but two of the controls were subsequently re-diagnosed as MS. All MS patients
had normal total protein levels and white cell counts. Three control patients had slightly raised total protein but normal white cell counts. One control (optic neuritis) had a white cell count of 26 (total protein normal) and a mirror pattern on IEF.

Table 2.3 Control patient demographics for *A. calcoaceticus* in MS study.

<table>
<thead>
<tr>
<th>Study number</th>
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<th>Sex</th>
<th>Diagnosis</th>
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<tbody>
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<td>Optic neuritis</td>
</tr>
<tr>
<td>C2</td>
<td>57</td>
<td>F</td>
<td>Progressive ataxia</td>
</tr>
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<td>23</td>
<td>F</td>
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</tr>
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<td>Chronic inflammatory demyelinating polyneuropathy</td>
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<td>55</td>
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<td>Unspecified brain neoplasm</td>
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Table 2.4 MS patient demographics for *A. calcoaceticus* in MS study.

<table>
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<th>Diagnosis</th>
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<td>M</td>
<td>Relapsing-remitting multiple sclerosis</td>
</tr>
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<td>MS3</td>
<td>57</td>
<td>F</td>
<td>Primary progressive multiple sclerosis</td>
</tr>
<tr>
<td>MS4</td>
<td>46</td>
<td>M</td>
<td>Primary progressive multiple sclerosis</td>
</tr>
<tr>
<td>MS5</td>
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<td>Relapsing-remitting multiple sclerosis</td>
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<tr>
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</table>

2.8 Enzyme-linked immunosorbent assay and precipitation methods

2.8.1 Turbidimetric assay for precipitation by sodium thiocyanate

Goat anti-human albumin (DiaSorin) was double diluted in 0.9% saline from 1/50 down to 1/3200. 50 µl of each dilution was pipetted into positions one to eleven across the first seven rows, A to G. The eighth row, H, had 50 µl of saline in positions one to eleven, to act as a control. Saline or NaSCN, 5M or 10M, was then added in varying amounts (see Table 2.5) and the plate read at 340 nm to measure any resulting turbidity, the saline acting as a control.
<table>
<thead>
<tr>
<th>Serum</th>
<th>Sal</th>
<th>Thio</th>
<th>Sal</th>
<th>Thio</th>
<th>Sal</th>
<th>Thio</th>
<th>Sal</th>
<th>Thio</th>
<th>Sal</th>
<th>Thio</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
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<td>25μl</td>
<td>50μl</td>
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<td>25μl</td>
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<td>50μl</td>
<td>100μl</td>
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</tr>
<tr>
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<td>5μl</td>
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<td>10μl</td>
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</tr>
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</tr>
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<td>100μl</td>
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<td>25μl</td>
<td>50μl</td>
<td>50μl</td>
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</tr>
<tr>
<td>Saline</td>
<td>5μl</td>
<td>5μl</td>
<td>10μl</td>
<td>10μl</td>
<td>25μl</td>
<td>25μl</td>
<td>50μl</td>
<td>50μl</td>
<td>100μl</td>
<td>100μl</td>
</tr>
</tbody>
</table>

Key: Sal, saline; Thio, sodium thiocyanate.

2.8.2 Enzyme-linked immunosorbent assay affinity distribution

Alternate rows (A, C, E and G) of a 96-well ELISA plate (Nunc) were incubated with 100 μl of 0.01% w/v HSA (10 mg/l) in 0.05M carbonate buffer at room temperature overnight, covered with clingfilm™. The wells were then washed out twice with 200 μl 0.9% saline before being incubated with 100 μl of 5M NaSCN for 15 minutes. The wells were then washed twice more with 0.9% saline before being blocked with 1% gelatin in 0.9% saline for 30 minutes on a shaking platform. 100 μl of goat anti-HSA diluted 1/250000 in 0.9% saline was then dispensed into each well and the plate incubated at room temperature on a platform shaker for one hour after which the plate was decanted. Saline and 10M NaSCN respectively, were then added in varying amounts to a final volume of 200 μl of 0M, 1.25M, 2.5M, 5M and 10M NaSCN in columns 1 and 2, 3 and 4, 5 and 6, 7 and 8 and 9 and 10, respectively, (see Table 2.6).

This enabled us to repeat all estimations in duplicate and the experiment in quadruplicate.
The plate was incubated with NaSCN for 30 minutes after which the NaSCN was decanted and the plate rinsed out with gently flowing tap water for approximately 30 seconds. The wells were then incubated with 100 μl of RAG serum conjugated to HRP, diluted 1/1000 in 0.9% saline/0.2% skimmed milk, on a platform shaker at room temperature for one hour. The wells were then washed four times with 200 μl of 0.1% gelatin/0.025% Tween 20/0.9% saline. 50 μl of o-phenylenediamine (OPD) solution was added to each well and the plate kept in the dark whilst the colour developed, apart from periodic checks to monitor colour development. When there was sufficient colour the colour reaction was stopped with 50 μl 1M HCl and the plate read at 490 nm, blanked at 405 nm, on a Victor2 1420 multilabel counter (Wallac). Affinity distribution was calculated by first subtracting the blank absorbance from every reading, then subtracting the net absorbances of the control (non-HSA-coated) wells from the net absorbances of the test (HSA-coated) wells before averaging the final duplicate absorbances at each different molarity of NaSCN. The absorbance at 0M NaSCN (saline) was taken as representing total specific IgG. The differences between the final

<table>
<thead>
<tr>
<th>Columns</th>
<th>Saline added (μl)</th>
<th>10M NaSCN added (μl)</th>
<th>Final molarity NaSCN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 and 2</td>
<td>200</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3 and 4</td>
<td>175</td>
<td>25</td>
<td>1.25</td>
</tr>
<tr>
<td>5 and 6</td>
<td>150</td>
<td>50</td>
<td>2.5</td>
</tr>
<tr>
<td>7 and 8</td>
<td>100</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>9 and 10</td>
<td>0</td>
<td>200</td>
<td>10</td>
</tr>
</tbody>
</table>
absorbances at each molarity of NaSCN were expressed as a percentage of total specific IgG using the following formulae according to the method of Luxton and Thompson (1990).

\[
\frac{(\text{OD}_{\text{saline}} - \text{OD}_{1.25 \text{M NaSCN}})}{\text{OD}_{\text{saline}}} \times 100
\]

\[
\frac{(\text{OD}_{1.25 \text{M NaSCN}} - \text{OD}_{2.5 \text{M NaSCN}})}{\text{OD}_{\text{saline}}} \times 100
\]

\[
\frac{(\text{OD}_{2.5 \text{M NaSCN}} - \text{OD}_{5 \text{M NaSCN}})}{\text{OD}_{\text{saline}}} \times 100
\]

\[
\frac{(\text{OD}_{5 \text{M NaSCN}} - \text{OD}_{10 \text{M NaSCN}})}{\text{OD}_{\text{saline}}} \times 100
\]

where \( \text{OD} \) = optical density \( i.e. \) absorbance

2.9 Rocket electrophoresis to quantitate serum and cerebrospinal fluid IgG

An agarose gel, 6 mm thick, (see Section 3.6) was prepared in advance and stored at 4 °C wrapped in clingfilm™ until use. The gel was placed on the cooling platten of an electrophoresis tank (Amersham) containing barbitone buffer and the wicks positioned to overlay the edge by approximately one centimetre. 4 μl of standards (50, 40, 30, 20, 10 and 5 mg/l), serum samples, (diluted 1 in 201), and CSF samples, (run neat and at 1 in 5 dilution) were pipetted, in duplicate, into two rows of wells cut into the gel. The gel was then run overnight at 150 V and an approximate current of 25 mA. The gel was removed from the tank and pressed under six sheets of filter paper, a glass plate and a 1 kg weight for ten minutes. The gel was submerged in 0.9% saline for 30 minutes before being pressed as before and then dried using a hairdrier. Next, the gel was submerged in stain, (Coomassie Brilliant Blue), for 30 minutes, after which it was rinsed in tap water, blotted as before and dried. Finally, the gel was placed in destain until the background was clear, washed in tap water, blotted and dried. Peak heights were measured and a standard curve constructed from which the concentration values of the unknowns were determined.
2.10 Statistical analyses

2.10.1 General Linear Model

The general linear model (GLM) is used to compare values from more than two groups of non-normally distributed data. Initial analysis gives an F value, which is used to determine overall significance. If $p<0.05$ then post hoc analysis by least squares means regression analysis is performed.

2.10.2 Mantel-Haenszel Chi Square

The Mantel-Haenszel chi-square is used to establish whether there is a linear association between more than two sets of variables. Significance in this test shows that increases in one variable are associated with increases in the other greater than would be expected by chance alone.

2.10.3 Fisher Exact Probability Test

The Fisher exact probability test is a non-parametric technique for analysing two small samples, the results of each observation being one of only two possible outcomes. Scores are given as frequencies and the test determines whether the two groups differ in the proportion of the frequencies of the two outcomes.
3.0 Materials

3.1 Buffers

3.1.1 Acetate buffer

**Stock acetate buffer (0.2M):** Dissolve 136 g of sodium acetate trihydrate (Sigma) and 22.5 ml of glacial acetic acid (BDH) in deionised water and adjust pH to 5.1, if necessary. Make up to 5 litres with deionised water.

**Working acetate buffer (0.02M):** Dilute stock acetate buffer 1 in 10 with deionised water.

3.1.2 Carbonate buffer

**Stock carbonate buffer (0.5M):** Dissolve 6.93 g of sodium carbonate (Sigma) and 13.05 g of sodium hydrogen carbonate (Sigma) in 500 ml of deionised water to make a 0.5M solution.

**Working carbonate buffer (0.05M):** Dilute stock carbonate buffer 1 in 10 with deionised water.

3.1.3 Barbitone buffer for rocket electrophoresis

10.5 g diethylbarbituric acid (Sigma), 65.0 g sodium barbitone (Sigma) and 2.0 g calcium lactate (Sigma) are dissolved in 2 l of distilled water. The solution is made up to 5 l with distilled water and 5 ml thymol (Sigma) solution added.
3.2 Sodium thiocyanate

Stock sodium thiocyanate solution (10M): Dissolve 202.68 g of NaSCN (Sigma) in 250 ml deionised water.

Working solutions (1.25M, 2.5M, 5M): 1.25M, add 3.125 ml of stock solution to 21.875 ml deionised water; 2.5M, add 6.25 ml stock solution to 18.75 ml deionised water; 5M, add 12.5 ml stock solution to 12.5 ml deionised water.

3.3 Saline solutions

0.9% Saline: Dissolve 90 g of sodium chloride (Sigma) in 10 litres of deionised water.

0.9% Saline/1mM EDTA: Dissolve 2 g of EDTA in 5 litres of 0.9% saline.

3.4 Electrode solutions for isoelectric focusing

Sodium hydroxide (1M) (catholyte): Dissolve 40 g sodium hydroxide (Sigma) in deionised water and make up to 1 litre.

Sulphuric acid (0.05M) (anolyte): Add 1.35 ml concentrated sulphuric acid (BDH) to 500 ml deionised water.

3.5 Colour reagents

3.5.1 3-amino-9-ethyl carbazole

Dissolve 50 mg of 3-amino-9-ethyl carbazole (Sigma) in 20 ml ethanol (Hayman) and add 100 ml working-strength acetate buffer (see section 3.1.1). Add 100 µl 30% v/v hydrogen peroxide just before use.
3.5.2 4-chloro-1-naphthol

Dissolve 0.25 mg of 4-chloro-1-naphthol in 10 ml ethanol; add 50 ml of 0.02M acetate buffer. Add 50 µl 30% v/v hydrogen peroxide (Sigma) just before use.

3.5.3 O-phenylenediamine

Dissolve 10 mg OPD (Sigma) in 10 ml 0.02M acetate buffer and add 12 µl of 30% v/v hydrogen peroxide just before use.

3.5.4 Coomassie Brilliant Blue and destain

Dissolve 2 g Coomassie Brilliant Blue (Sigma) in 350 ml ethanol. When the stain has dissolved add 550 ml distilled water and 100 ml glacial acetic acid (BDH). Destain solution is 550 ml distilled water, 350 ml ethanol and 100 ml glacial acetic acid.

3.6 Agarose gel for rocket electrophoresis

In a glass, screwtop bottle dissolve 6 g PEG 6000 (BDH) in 200 ml of barbitone buffer (see Section 3.1.3) and add 2 g high electroendosmosis agarose (HEEO) (BMA Products) and 1 g medium electroendosmosis agarose (MEEO) (BMA Products). Place in a boiling water bath until the gel has fully dissolved, then aliquot 25 ml volumes into glass, screwtop bottles and store at 4 °C. To make a gel for IgG rocket electrophoresis take one of the 25 ml aliquots and place into a boiling water bath until dissolved. Then transfer the bottle to a water bath at 65 °C for 30 minutes to allow the gel to cool to 65 °C. Cut a sheet of GelBond to 23 cm x 12 cm and seal on the surface of a glass plate using 50:50 water: methanol. On top place a second glass plate with attached spacer of 6 mm depth and clamp the apparatus together. Warm gently under a hairdrier. Add 80 µl of goat anti-human IgG Fc (Dako, ) and mix gently to avoid frothing. Using a warm
syringe and needle transfer gel into casting frame. When set, the gel is wrapped in clingfilm™ and stored for up to one week at 4 °C.

3.7 Antibodies and their dilutions

Model method antibodies:

First antibody – rabbit anti-goat (RAG) polyclonal (Dako) diluted 1 in 2500 i.e. 10 μl of antibody in 25 ml of 0.2% w/v skimmed milk in 0.9% saline.

Second antibody – swine anti-rabbit polyclonal conjugated with horseradish peroxidase (SAR-HRP) (Dako) diluted 1 in 5000 i.e. 5 μl of antiserum in 25 ml of 0.2% w/v skimmed milk in 0.9% saline.

Band comparison in SSPE study and affinity maturation study in HSE antibodies:

First antibody – goat anti-human IgG (Diasorin) diluted 1 in 2500 i.e. 10 μl of antibody in 25 ml of 0.2% w/v skimmed milk in 0.9% saline.

Second antibody - RAG conjugated with HRP (RAG-HRP) (Dako) diluted 1 in 5000 i.e. 5 μl of antibody in 25 ml of 0.2% w/v skimmed milk in 0.9% saline.

Acinetobacter calcoaceticus in MS study antibodies:

First antibody – goat anti-human IgG (Diasorin) diluted 1 in 2500 i.e. 10 μl of antibody in 25 ml of 0.2% w/v skimmed milk in 0.9% saline/1mM EDTA/0.1% v/v Tween 20.

Second antibody – RAG conjugated with HRP (Dako) diluted 1 in 5000 i.e. 5 μl of antibody in 25 ml of 0.2% w/v skimmed milk in 0.9% saline/1mM EDTA/0.1% v/v Tween 20.
Chlamydophila pneumoniae in MS study antibodies for Western blotting:
Rabbit anti-human IgG, IgM, IgA, κ light chain and λ light chain conjugated to HRP (Dako) diluted 1/1000 i.e. 25 μl antibody in 25 ml 0.9% saline/0.2% w/v skimmed milk for each manifold.

ELISA for affinity distribution:
First antibody – goat anti-human albumin (Diasorin) diluted 1/250000 i.e. stock solution of 10 μl in 250 ml 0.9% saline further diluted 1/10 i.e. 10 ml stock made up to 100 ml with 0.9% saline.
Second antibody - RAG polyclonal conjugated to HRP, diluted 1/1000 i.e. 25 μl antibody in 25 ml 0.9% saline/0.2% skimmed milk.

3.8 Software applications used

3.8.1 Irfanview
Irfanview is a freeware graphics viewer for Windows developed by Irfan Skiljan at the University of Vienna. Version 3.51 was used.

3.8.2 ImageJ
ImageJ is a freeware Java-based program for image processing and analysis developed at the NIH, USA.
4.0 Results

4.1 Model method

4.1.1 Model method results

The aim of the model method was to show, using commercial antigen and antisera, that the method could be used to investigate the relative affinity of individual clones. The final blots had to be of sufficient quality for them to be scanned and subjected to the software analysis.

On each blot, six replicates of commercial goat anti-HSA, diluted 1/1000 in 0.9% saline, and isoelectrically focused, were incubated with each of the four different molarities of NaSCN. After development, blots were dried and scanned ready for peak analysis. An example is given (Figure 4.1).

For all subsequent images of blots the cathodal edge is at the top of the image.

For all images of densitograms the cathodal end is at the left of the image.

![Figure 4.1 Complete trace of model method blot 3.](image)
One representative band was identified as being of sufficient strength to be the "target" band (Figure 4.2). The pixel density of this band was then calculated for each trace in each assay and used to generate the statistics (Figure 4.3).

Figure 4.2 Examples of IEF trace from model method blot 18.

Key: A, 0M; B, 1.25M; C, 2.5M; D, 5M NaSCN. Representative high affinity band is indicated with arrow.

Some traces were not suitable for peak analysis due to problems that occurred during the running of the gel; principally, skewed traces that could not be accommodated by the software and traces with discrete areas of inappropriate colour development, both too heavy and too light. All observations were made on at least three measurements, as indicated in Table 4.1.
Figure 4.3 Densitograms of IEF traces from Figure 4.2.

Key: A, 0M; B, 1.25M; C, 2.5M; D, 5M NaSCN. Peak outlined is the peak calculated as a percentage of total area. Plots are not shown at relative scale.

Twenty blots were run, of which two were unsuitable for any analysis due to skewed traces. Of the remaining eighteen, five were rejected on the basis that there were not three or more measurable replicates at each molarity. The results of these thirteen complete blots (Table 4.1, Figures 4.4 and 4.5) were used to test for further statistical analysis.

Little difference can be seen between the overall values from 0M, 1.25M and 2.5M NaSCN although there is an upward trend, which is reflected, in most of the individual sets of values, i.e. 10/13 values at 5M are above the highest value of the 0M group.
Table 4.1 Peak area of target band (pixel density)

<table>
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<tr>
<th>ID</th>
<th>Molarity of sodium thiocyanate</th>
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<th>1.25M</th>
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<th>5M</th>
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<tbody>
<tr>
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</tr>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>(n)</td>
<td>%</td>
<td>(n)</td>
</tr>
<tr>
<td>All</td>
<td></td>
<td>2.8 ± 1.3</td>
<td>46.1</td>
<td>3.7 ± 2.0</td>
<td>52.6</td>
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<td>(13)</td>
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<td>(13)</td>
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<tr>
<td>1</td>
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<td>(6)</td>
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<td>2</td>
<td>5.4 ± 0.4</td>
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<td>(6)</td>
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<td>(4)</td>
<td>(4)</td>
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<td>(6)</td>
</tr>
<tr>
<td>6</td>
<td>3.3 ± 0.9</td>
<td>27.3</td>
<td>3.7 ± 0.8</td>
<td>22.2</td>
<td>16.5</td>
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<td>(6)</td>
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<td>4.9</td>
<td>3.5 ± 0.2</td>
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<td>7.9</td>
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<td>(6)</td>
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<td>2.5 ± 0.1</td>
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<td>(3)</td>
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<td>(5)</td>
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<td>1.3 ± 0.2</td>
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<td>14.1</td>
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<td>(6)</td>
<td>(3)</td>
<td>(6)</td>
</tr>
<tr>
<td>14</td>
<td>2.1 ± 0.3</td>
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<td>2.5 ± 0.4</td>
<td>18.0</td>
<td>8.5</td>
</tr>
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<td>(6)</td>
<td>(6)</td>
<td>(6)</td>
</tr>
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<td>1.4 ± 0.1</td>
<td>3.6</td>
<td>2.5 ± 0.4</td>
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<td>21.4</td>
</tr>
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<td>(3)</td>
<td>(6)</td>
<td>(6)</td>
<td>(6)</td>
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<tr>
<td>16</td>
<td>1.1 ± 0.2</td>
<td>20.4</td>
<td>1.7 ± 0.2</td>
<td>8.6</td>
<td>19.6</td>
</tr>
<tr>
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<td>(6)</td>
<td>(3)</td>
<td>(5)</td>
<td>(6)</td>
</tr>
<tr>
<td>17</td>
<td>3.1 ± 0.4</td>
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<td>4.4 ± 0.7</td>
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<td>(6)</td>
<td>(3)</td>
<td>(4)</td>
<td>(6)</td>
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<tr>
<td>18</td>
<td>2.2 ± 0.3</td>
<td>14.0</td>
<td>3.0 ± 0.3</td>
<td>10.6</td>
<td>30.8</td>
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<tr>
<td></td>
<td></td>
<td>(6)</td>
<td>(4)</td>
<td>(4)</td>
<td>(6)</td>
</tr>
</tbody>
</table>

Areas expressed as a percentage of total area under curve.

Values given ± standard deviation with number of observations, (n), in brackets.
4.1.1.1 Statistical analysis

There was a significant difference in the pixel density of the chosen peak F (3, 48) = 11.81, p < 0.0001. The subsequent post hoc analysis by multiple squares regression analysis revealed that this was due to a significant higher relative pixel density at the 5M concentration compared to the 0M, 1.25M and 2.5M concentrations of NaSCN. No direct quantitative statistical difference could be demonstrated between the 0M, 1.25M and 2.5M concentrations (Table 4.2).

Table 4.2 Results of post hoc statistical analysis by multiple squares regression analysis of comparisons of pixel density between NaSCN incubations in model method.

<table>
<thead>
<tr>
<th>Molarity of NaSCN</th>
<th>0M</th>
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<th>2.5M</th>
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<tbody>
<tr>
<td>0M</td>
<td>NS</td>
<td>NS</td>
<td>p&lt;0.0001</td>
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<td>1.25M</td>
<td>NS</td>
<td>NS</td>
<td>p&lt;0.0001</td>
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</tr>
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<td>2.5M</td>
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<td>p=0.0002</td>
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</tr>
<tr>
<td>5M</td>
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<td>p&lt;0.0001</td>
<td>p=0.0002</td>
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</tr>
</tbody>
</table>

Key: Significant values are shown in bold italic; non-significant values are indicated by NS (Not Significant).

However, on a categorical level, comparing proportions of samples above and below cut-off, the trend analysis also demonstrated a significant increase ($\chi^2 = 18.13$, p < 0.001) in relative pixel density with increasing concentrations of NaSCN. The cut-off value was defined as the highest value within the 0M set of observations, which was 5.4%, therefore the 0M group contained 0/13 values above cut-off i.e. 0%. In the 1.25M group 2 of 13 values were above the cut-off, equating to 15%. This figure then doubled to 4/13 in the 2.5M group, 31%, and was further increased to 10/13 in the 5M group (77%).
In order to show that it is antigen-specific high affinity antibody pixel densities were compared, derived from the densitogram, of the target band with underlying polyclonal background (band/polyclonal) with an area of polyclonal IgG (see Figure 4.6) for blots 3 and 18. Both measured areas were 25 pixels wide and the two were separated by 60 pixels. Measurements were always begun from the bottom left of the target peak. At each molarity six measurements were taken and averaged for band/polyclonal and polyclonal areas; the ratio being generated by dividing the band/polyclonal area pixel density by the polyclonal area pixel density (Table 4.3).

![Figure 4.4 Line plots of all blot values for model method.](image)

**Figure 4.4** Line plots of all blot values for model method.
Figure 4.5 Average values of all blots for model method.

Error bars are 1SD above and below.

Figure 4.6 Areas used for band/polyclonal and polyclonal ratio in model method.

Key: A, 0M; B, 1.25M; C, 2.5M; D, 5M NaSCN.
Table 4.3 Ratio of band/polyclonal to polyclonal pixel density.

<table>
<thead>
<tr>
<th>NaSCN (M)</th>
<th>0</th>
<th>1.25</th>
<th>2.5</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blot 3</td>
<td>2.29</td>
<td>2.56</td>
<td>2.45</td>
<td>12.80</td>
</tr>
<tr>
<td>Blot 18</td>
<td>2.36</td>
<td>2.07</td>
<td>2.34</td>
<td>16.03</td>
</tr>
</tbody>
</table>

This shows that while there is little difference at 0, 1.25 and 2.5M NaSCN, at 5M NaSCN the ratio rises markedly, confirming that the polyclonal background is diminished in proportion to the bands of antigen-specific IgG, which are of higher affinity.

4.1.2 Blocking the membrane

Ultrabind (UB450) membrane is a covalent binding membrane containing active aldehyde groups that bind free amino groups, thus binding proteins to the membrane. Solutions containing primary amines could therefore be used to block any available binding sites once the antigen of interest has been bound to the membrane. The manufacturer’s recommendations for blocking the membrane included 1% skimmed milk in saline, which was initially adopted, although subsequently 2% skimmed milk in saline was used according to normal practice in the laboratory.

Experiments with anti-human albumin serum blotted onto a membrane coated only with milk proteins showed faint patterns indicating that the blocking may not have been complete. A similar experiment blocking the membrane with 1% Tris in saline also failed to block the membrane completely (Figure 4.7). A combination of 1% Tris and 2% skimmed milk in saline was successful in completely blocking the membrane and was subsequently adopted for all work with Ultrabind membrane (Figure 4.8). Anti-human albumin serum was double diluted from 1/500 to 1/16000 for these experiments.
Figure 4.7 Goat anti-HSA blotted against Tris-coated membrane and incubated with sodium thiocyanate.

Figure 4.8 Goat anti-HSA blotted against milk/Tris-coated membrane and incubated with sodium thiocyanate.
4.1.3 Enzyme-linked immunosorbent assay affinity distribution for anti-HSA

The aim of this ELISA was to show, independently of affinity immunoblotting, that goat anti-HSA serum did contain antibodies of sufficient affinity for HSA to resist elution by 5M NaSCN.

The relative affinities were 100.0% at 0M NaSCN (saline), 41.9% at 1.25M NaSCN, 38.1% at 2.5M NaSCN, 10.5% at 5M NaSCN and 7.6% at 10M NaSCN.

The results show that there is high affinity antibody present in the commercial goat anti-HSA serum. 18.1% of the total anti-albumin IgG in the sample is of sufficiently high affinity to resist at least 5M NaSCN (Figure 4.9).

![Affinity distribution of anti-HSA](image)

**Figure 4.9 Affinity distribution histogram for anti-HSA serum.**

4.2 Comparison of band in serum and cerebrospinal fluid in subacute sclerosing panencephalitis

A paired serum and CSF sample was taken from a patient routinely admitted to NHNN, known to be suffering from SSPE. This was a confirmed diagnosis, with a specific measles IgG index of 71.13, assayed at Colindale Public Health Laboratory. Serum anti-
measles IgG was measured at 13164 mIU/ml, serum albumin 40 g/l, whilst CSF anti-measles IgG was 1264 mIU/ml, with CSF albumin of 54 mg/l. The index was thus calculated as:

\[
\frac{\text{CSF IgG}}{\text{serum IgG}} \times \frac{\text{serum albumin}}{\text{CSF albumin}}
\]

i.e.

\[
\frac{1264}{13164} \times \frac{40000}{54} = 71.13
\]

Routine IEF in this laboratory showed the patient to have a “greater than” pattern (Anderssen et al, 1994) with the CSF pattern showing many more bands, and which were considerably stronger. Routine antigen immunoblotting revealed a similar pattern (see Figure 4.10).

![Figure 4.10 Routine blots of SSPE patient.](image)

Key: S, serum; C, CSF.

Measurements of total IgG in serum (9.35 g/l) and CSF (88 mg/l), by rocket electrophoresis, gave a ratio of 1:106.25 which was used as the dilution factor in the
experiment. Three replicates of serum and three of CSF of the SSPE sample at each of the four molarities of thiocyanate (0M, 1.25M, 2.5M and 5M) were run (see Table 4.4).

**Table 4.4 Dilutions and final IgG concentrations for SSPE sample.**

<table>
<thead>
<tr>
<th>NaSCN molarity</th>
<th>Serum dilution</th>
<th>Total IgG (mg/l)</th>
<th>CSF dilution</th>
<th>Total IgG (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2124</td>
<td>4.4</td>
<td>20</td>
<td>4.4</td>
</tr>
<tr>
<td>1.25</td>
<td>1062</td>
<td>8.8</td>
<td>10</td>
<td>8.8</td>
</tr>
<tr>
<td>2.5</td>
<td>1062</td>
<td>8.8</td>
<td>10</td>
<td>8.8</td>
</tr>
<tr>
<td>5</td>
<td>106.3</td>
<td>88</td>
<td>Neat</td>
<td>88</td>
</tr>
</tbody>
</table>

The experiment was controlled with three clinically diagnosed paired MS samples, diluted to the same concentrations of IgG as in the SSPE sample, blotted against a measles-coated membrane (see Figure 4.12). The first control sample was from a patient with primary progressive MS, a “greater than” pattern on routine IEF serum IgG of 5.9 g/l and CSF IgG of 87 mg/l. The second and third were both patients with relapsing-remitting MS with routine IEF patterns showing bands in CSF, but not in serum. The second patient had serum IgG of 15.5 g/l and CSF IgG of 44 mg/l and the last control patient had serum IgG of 5.9 g/l and CSF IgG of 45 mg/l. The last two control CSF samples were applied at 8 µl, the first application of 4 µl being allowed to soak in before the second application of 4 µl. Finally, all four samples were appropriately diluted and blotted against a membrane coated only with control antigen, the control antigen being a freeze-dried preparation of the cell line that the virus is grown in (see Figure 4.11).
Figure 4.11 Three MS controls and SSPE sample against measles control antigen.

Key: SP, SSPE sample; M, MS sample; S, serum; C, CSF.

Figure 4.12 Three MS controls against measles antigen.

Key: MS, MS patient; S, serum; C, CSF.
A representative SSPE peak was identified (see Figure 4.13; arrowed, peak A) and its pixel density determined as a percentage of the pixel density of an area of the trace around it (see Figure 4.13; box). It was not measured as a percentage of the whole trace due to areas of saturation that were unavoidable even as a result of the dilutions. The trace at 5M NaSCN (see Figure 4.13) has become blurred and unreadable due to the presumed effect of high-strength thiocyanate.

![Figure 4.13 Paired sample of SSPE patient after exposure to NaSCN.](image)

Key: S, serum; C, CSF.
From the densitograms it can be seen that IgG is lost from the cathodal end (left) of the trace in both serum and CSF, as indicated in the area marked with X (see Figure 4.14). In serum, it is lost mainly with incubation in 1.25M NaSCN whereas in the CSF some is lost at 1.25M but most is stripped at 2.5M NaSCN. Visual examination of the peaks A, B and C show that in serum the peak heights remain in approximately the same ratio throughout, but in CSF peak A is taller than its neighbours, which retain their ratio. This suggests that CSF contains more high affinity IgG than serum and that peak A corresponds to a band of higher concentration in CSF than serum. Unfortunately, the traces at 5M NaSCN could not be studied due to interference.

Figure 4.14 Densitograms of SSPE samples.
Not shown to relative scale.
Table 4.5 Areas of band A used in comparison study on SSPE patient.

<table>
<thead>
<tr>
<th>Molarity</th>
<th>Type</th>
<th>Pixel Density</th>
<th>Mean + SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0M NaSCN</td>
<td>Serum</td>
<td>5.0</td>
<td>5.6 + 0.85</td>
</tr>
<tr>
<td></td>
<td>CSF</td>
<td>10.3</td>
<td>10.2 + 0.85</td>
</tr>
<tr>
<td>1.25M</td>
<td>Serum</td>
<td>7.1</td>
<td>6.9 + 0.15</td>
</tr>
<tr>
<td>NaSCN</td>
<td>CSF</td>
<td>11.6</td>
<td>10.7 + 1.25</td>
</tr>
<tr>
<td>2.5M</td>
<td>Serum</td>
<td>6.9</td>
<td>11.6 + 4.10</td>
</tr>
<tr>
<td>NaSCN</td>
<td>CSF</td>
<td>21.0</td>
<td>25.6 + 4.29</td>
</tr>
</tbody>
</table>

The data show that the serum band, on average is 2.1 times higher at 2.5M NaSCN than at 0M, whilst the CSF band, on average, is 2.5 times higher over the same interval (Table 4.5, Figure 4.15).

Comparisons could not be made across the range of molarities as samples were diluted differently at 1.25 and 2.5M as opposed to 0M (see Table 4.4). No results could be obtained from the 5M NaSCN traces due to repeatedly poor quality of the traces because of the interference effects of high molarity thiocyanate (Gray and Shaw, 1992; McMahon and Kennedy, 2000; Bouvet et al, 2001a; Bouvet et al, 2001b).

In order to see whether there were any significant differences between the three replicate serum and three replicate CSF results at each molarity categorical data analysis was used. To enable a categorical data analysis the highest pixel density observed for serum samples was defined as cut-off (pixel density = 6.6 for 0M, pixel density = 7.1 for 1.25M, pixel density = 14.1, 2.5M).
Figure 4.15 Pixel density of target band in SSPE patient serum and CSF.

Error bars are 1SD

A significantly higher proportion of CSF samples (100%, 3/3) had pixel density levels above cut-off compared to the serum samples (0%, 0/3) for each of the comparisons (p<0.05, Fisher’s exact test).

The area of the target band plus the polyclonal background beneath it, (band/polyclonal), was compared with an area of polyclonal background, both of width 20 pixels, across all the molarities, to calculate the ratio of band/polyclonal to polyclonal (see Table 4.6, boxed areas in Figure 4.16) in CSF. This could not be done in the serum as there was no signal in the corresponding polyclonal area. The ratio was calculated by dividing the band/polyclonal pixel density by the polyclonal pixel density.

Table 4.6 Band/polyclonal to polyclonal ratios for target band in SSPE CSF.

<table>
<thead>
<tr>
<th>NaSCN (M)</th>
<th>0</th>
<th>1.25</th>
<th>2.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio</td>
<td>0.91</td>
<td>1.30</td>
<td>1316</td>
</tr>
</tbody>
</table>
The ratio shows little change between 0M and 1.25M NaSCN but climbs to 1316 at 2.5M due to the absence of any signal in the polyclonal area. A nominal value of 1 was therefore applied to prevent division by zero.

Figure 4.16 Band/polyclonal and polyclonal areas for ratio calculation in SSPE.

4.3 Affinity maturation in Herpes simplex encephalitis

A series of three paired serum and CSF samples was obtained from a 68 year old female admitted to the NHNN suffering from suspected encephalitis. The first paired sample was taken the day after admission and showed 200 lymphocytes/mm³ in the CSF, a mirror pattern on routine IEF, albeit with subtle bands, stronger in CSF than serum, and positive for Herpes simplex type 1 by PCR for DNA. The mirror pattern was confirmed by staining separately for κ and λ light chains as the CSF trace was stronger, due to the raised total IgG. The patient was started on acyclovir therapy on admission. The second paired sample was from 6 days after admission and had the same IEF result for OCBs and a reported white cell count of 190 white cells/mm³, which were predominantly lymphocytes. PCR for DNA was now negative. The final sample, 15 days after
admission, was again negative for viral DNA by PCR and the white cell count had dropped to 36 white cells/ mm$^3$. IEF for OCBs now showed a pattern of more bands in the CSF than in the serum (Table 4.7).

Total IgG was calculated for all samples by rocket electrophoresis. This enabled all samples to be diluted to 26, 52 and 104 mg/l total IgG for three complete affinity blots.

<table>
<thead>
<tr>
<th>Table 4.7 Results summary for serial samples on patient with HSE.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First sample</strong></td>
</tr>
<tr>
<td><strong>Day after admission</strong></td>
</tr>
<tr>
<td><strong>Serum IgG (mg/l)</strong></td>
</tr>
<tr>
<td>NR 5000 to 10000</td>
</tr>
<tr>
<td><strong>CSF IgG (mg/l)</strong></td>
</tr>
<tr>
<td>NR 5 to 25</td>
</tr>
<tr>
<td><strong>PCR for HSV1 DNA</strong></td>
</tr>
<tr>
<td><strong>White cell count (mm$^3$)</strong></td>
</tr>
<tr>
<td><strong>Total protein (g/l)</strong></td>
</tr>
<tr>
<td>NR 0.10 to 0.60</td>
</tr>
</tbody>
</table>

Key: NR, normal range.
At all three dilutions the day 1 paired sample had only a few faint bands compared with the days 6 and 15 paired samples although more bands could be seen at 104 mg/l IgG than at 26 mg/l IgG. There was also a notable increase in the polyclonal background as the sample dilutions decreased. At all dilutions the bands in the day 1 sample were lost at 5M NaSCN whilst they were plainly visible for the days 6 and 15 paired samples. At 52 mg/l total IgG the bands could not be reliably distinguished at 5M NaSCN due to presumed high molarity thiocyanate interference. Interestingly, this was the latest of the experiments on this series, the blot at 104 mg/l being run first, the blot using 26 mg/l run five days later and the blot using 52 mg/l six days after that. All of the paired samples were controlled by blotting against herpes control antigen (see Figure 4.17). It was decided to use the blot of 23 mg/l for the subsequent work (Figure 4.18).
Figure 4.18 Blot of HSE series at 26 mg/l.

Key: Arrows indicate three bands quantified, identified as A (top), B (middle) and C (bottom). S, serum; C, CSF.

Table 4.8 Pixel density of total area under the curve for HSE series.

<table>
<thead>
<tr>
<th>NaSCN (M)</th>
<th>0M</th>
<th>1.25M</th>
<th>2.5M</th>
<th>5M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1 Serum</td>
<td>5705</td>
<td>0</td>
<td>172</td>
<td>2781</td>
</tr>
<tr>
<td>Day 1 CSF</td>
<td>8312</td>
<td>100</td>
<td>1030</td>
<td>844</td>
</tr>
<tr>
<td>Day 6 Serum</td>
<td>12356</td>
<td>6385</td>
<td>6509</td>
<td>2954</td>
</tr>
<tr>
<td>Day 6 CSF</td>
<td>27394</td>
<td>29779</td>
<td>25056</td>
<td>11846</td>
</tr>
<tr>
<td>Day 15 Serum</td>
<td>17139</td>
<td>15506</td>
<td>10482</td>
<td>6368</td>
</tr>
<tr>
<td>Day 15 CSF</td>
<td>51306</td>
<td>46828</td>
<td>36807</td>
<td>13839</td>
</tr>
</tbody>
</table>
### Table 4.9 Pixel densities for the target bands at day 6 of HSE series.

<table>
<thead>
<tr>
<th>NaSCN (M)</th>
<th>Sample</th>
<th>Band A (%)</th>
<th>Band B (%)</th>
<th>Band C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Serum</td>
<td>3.6</td>
<td>1.5</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>CSF</td>
<td>0.8</td>
<td>1.0</td>
<td>1.7</td>
</tr>
<tr>
<td>1.25</td>
<td>Serum</td>
<td>11.4</td>
<td>6.8</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>CSF</td>
<td>1.0</td>
<td>1.1</td>
<td>1.7</td>
</tr>
<tr>
<td>2.5</td>
<td>Serum</td>
<td>14.2</td>
<td>9.5</td>
<td>9.9</td>
</tr>
<tr>
<td></td>
<td>CSF</td>
<td>2.5</td>
<td>1.7</td>
<td>1.9</td>
</tr>
<tr>
<td>5</td>
<td>Serum</td>
<td>18.6</td>
<td>16.2</td>
<td>15.3</td>
</tr>
<tr>
<td></td>
<td>CSF</td>
<td>5.2</td>
<td>4.0</td>
<td>5.7</td>
</tr>
</tbody>
</table>

Areas expressed as percentage of total area under the curve.
Key: S, serum; C, CSF.

Three bands, as indicated in Figure 4.18, were quantified using ImageJ on a digitised blot for day 6 and day 15 samples (Tables 4.9 and 4.10, Figures 4.20 and 4.21). No data could be extracted from the digitised blot for the first sample.

### Table 4.10 Pixel densities for the target bands at day 15 of HSE series

<table>
<thead>
<tr>
<th>NaSCN (M)</th>
<th>Sample</th>
<th>Band A (%)</th>
<th>Band B (%)</th>
<th>Band C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Serum</td>
<td>3.1</td>
<td>1.9</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>CSF</td>
<td>0.8</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>1.25</td>
<td>Serum</td>
<td>4.0</td>
<td>2.9</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>CSF</td>
<td>0.8</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>2.5</td>
<td>Serum</td>
<td>6.6</td>
<td>3.9</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>CSF</td>
<td>1.4</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>Serum</td>
<td>16.0</td>
<td>9.7</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td>CSF</td>
<td>4.1</td>
<td>3.7</td>
<td>3.1</td>
</tr>
</tbody>
</table>

Areas expressed as percentage of total area under the curve.
Key: S, serum; C, CSF.

The total pixel density for each trace at 26 mg/l (Figure 4.18) was plotted against time (Figure 4.19) to show the increase in total specific IgG.
Figure 4.19 Plot of total pixel density against time for HSE.

Figure 4.20 Target bands on day 6 in HSE patient.

Figure 4.21 Target bands on day 15 in HSE patient.
Figure 4.22 Densitograms of day 6 sample in HSE patient.

Figure 4.23 Densitograms of day 15 sample in HSE patient.
Looking at the area marked X (see Figures 4.22 and 4.23), anodal to peak C, on the densitograms it was seen that at day 6, the IgG here is largely lost in the serum at 1.25M NaSCN and completely at 2.5M NaSCN, suggesting this IgG to be of low affinity. In the CSF, the same area shows little difference at 1.25M, is reduced at 2.5M NaSCN but is not lost until 5M NaSCN is applied. The findings are the same for CSF on day 15, but in serum there is still IgG present at 1.25m NaSCN. Only at 2.5M NaSCN is the IgG virtually absent from the area. This shows that whilst the IgG in the CSF is of higher affinity than in the serum at days 6 and 15, the serum IgG is of higher affinity at day 15 than day 6.

In the serum, the largest rise at day 6 comes from 0M to 1.25M NaSCN, whereas in CSF, the largest rise is from 2.5M to 5M NaSCN. By day 15, both serum and CSF show the largest rise from 2.5M to 5M.

The plot of total pixel density under the curve shows rises in both serum and CSF over time (Table 4.8). As would be expected, as the molarity of the NaSCN rises, the total pixel density drops as lower affinity IgG is removed.

---

**Figure 4.24 Band/polyclonal and polyclonal areas for ratio calculation in HSE.**
To further underline the point that it is antigen-specific high affinity antibody the pixel densities were compared, on the densitogram, of an area corresponding to the peak A and underlying polyclonal background (band/polyclonal) and an area of polyclonal IgG (see Figure 4.24). Each area was 20 pixels wide and the two were separated by 50 pixels. Dividing the band/polyclonal area pixel density by the polyclonal area a ratio was generated. The figures (see Table 4.11) confirm that at day 1 there was no detectable high affinity antigen-specific IgG, there being no detectable signal in the areas at any molarity of NaSCN. At both day 6 and day 15 the ratio climbed (except between 1.25M and 2.5M for CSF), as the molarity of NaSCN increased, the ratios being greatly raised at 5M.

<table>
<thead>
<tr>
<th></th>
<th>0M</th>
<th>1.25M</th>
<th>2.5M</th>
<th>5M</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 1 serum</strong></td>
<td>18.10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Day 1 CSF</strong></td>
<td>2.82</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Day 6 serum</strong></td>
<td>1.95</td>
<td>7.45</td>
<td>19.85</td>
<td>1008*</td>
</tr>
<tr>
<td><strong>Day 6 CSF</strong></td>
<td>2.47</td>
<td>1.81</td>
<td>1.52</td>
<td>6.25</td>
</tr>
<tr>
<td><strong>Day 15 serum</strong></td>
<td>1.85</td>
<td>2.62</td>
<td>4.05</td>
<td>338.00</td>
</tr>
<tr>
<td><strong>Day 15 CSF</strong></td>
<td>1.35</td>
<td>1.73</td>
<td>1.52</td>
<td>5.58</td>
</tr>
</tbody>
</table>

* Nominal value of 1 given to polyclonal area to avoid division by zero.

4.4 Acinetobacter calcoaceticus in MS

The production of oligoclonal, polyspecific immunoglobulin G (IgG) is characteristic of MS, yet no pathogen has been identified as an infectious agent (Talbot et al, 2001; Steiner et al, 2001). Recent studies (Hughes et al, 2001; Hughes et al, 2003) have proposed *Acinetobacter calcoaceticus* and *Pseudomonas aeruginosa* as candidate organisms, on the basis of a sequence homology between a bacterial enzyme and bovine
MBP (Eylar et al, 1970). To investigate this specific, high affinity IgG against these pathogens was looked for in paired serum and cerebrospinal fluid (CSF) from MS patients compared to other neurological diseases.

The aims of this study were to determine whether MS patients possess antibodies against Acinetobacter calcoaceticus, Pseudomonas aeruginosa, Escherichia coli and Proteus mirabilis in their CSF and serum, compared to control patients with other neurological diseases. Controls included inflammatory diseases of the CNS to assess whether any antibodies observed were of high affinity. Proteus mirabilis and Escherichia coli were included as control organisms; Proteus mirabilis as a classic enteropathogen and Escherichia coli as it also contains the encephalitogenic sequence within its proteins, although not in the same protein. This was carried out using the technique of antigen immunoblotting, which involves separating sample IgG by IEF and then blotting onto a membrane impregnated with antigen to capture the antibodies of interest (Moyle et al, 1984).

Table 4.12 Results for control patients in A. calcoaceticus in MS study.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>IEF pattern of total IgG in serum and CSF</th>
<th>IEF of E. coli-specific IgG in serum and CSF</th>
<th>IEF of P. mirabilis-specific IgG in serum and CSF</th>
<th>IEF of P. aeruginosa-specific IgG in serum and CSF</th>
<th>IEF of A. calcoaceticus-specific IgG in serum and CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>&gt; Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>C2</td>
<td>&gt; Mirror (2.5)</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>C3</td>
<td>* Mirror (2.5)</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>C4</td>
<td>* Negative</td>
<td>Mirror (2.5)</td>
<td>Mirror (2.5)</td>
<td>Mirror (2.5)</td>
<td>Mirror (2.5)</td>
</tr>
<tr>
<td>C5</td>
<td>+ Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Mirror (5)</td>
<td>Negative</td>
</tr>
<tr>
<td>C6</td>
<td>- Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>C7</td>
<td>* Negative</td>
<td>Mirror (2.5)</td>
<td>Negative</td>
<td>Mirror (2.5)</td>
<td>Negative</td>
</tr>
<tr>
<td>C8</td>
<td>+ Mirror (2.5)</td>
<td>Negative</td>
<td>Mirror (2.5)</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Bracketed figure indicates highest molarity of NaSCN at which bands present
Table 4.13 Results for MS patients in *A. calcoaceticus* in MS study.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>IEF pattern</th>
<th>IEF of <em>E. coli</em>-specific IgG in serum and CSF</th>
<th>IEF of <em>P. mirabilis</em>-specific IgG in serum and CSF</th>
<th>IEF of <em>P. aeruginosa</em>-specific IgG in serum and CSF</th>
<th>IEF of <em>A. calcoaceticus</em>-specific IgG in serum and CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS1</td>
<td>&gt;&gt;</td>
<td>Negative</td>
<td>Mirror (2.5)</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>MS2</td>
<td>&gt;&gt;</td>
<td>Mirror (2.5)</td>
<td>Mirror (2.5)</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>MS3</td>
<td>&gt;&gt;</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>MS4</td>
<td>&gt;&gt;</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>MS5</td>
<td>&gt;</td>
<td>Mirror (2.5)</td>
<td>Negative</td>
<td>Mirror (2.5)</td>
<td>Negative</td>
</tr>
<tr>
<td>MS6</td>
<td>+</td>
<td>Negative</td>
<td>Negative</td>
<td>Mirror (1.25)</td>
<td>Negative</td>
</tr>
<tr>
<td>MS7</td>
<td>+</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>MS8</td>
<td>+</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>MS9</td>
<td>+</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>MS10</td>
<td>+</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>MS11</td>
<td>+</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive (2.5)</td>
</tr>
<tr>
<td>MS12</td>
<td>&gt;&gt;</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Bracketed figure indicates highest molarity of NaSCN at which bands present

*Acinetobacter calcoaceticus*. Of the eight control samples, three had detectable band patterns against *A. calcoaceticus*, all of them mirror patterns. Two of the mirror patterns were visible after incubation with 2.5M NaSCN and the other after incubation with 5M NaSCN. Only one of the MS patients had specific bands against *Acinetobacter calcoaceticus*, still visible after incubation with 2.5M NaSCN, which appeared stronger in CSF than serum (Figure 4.25).

*Pseudomonas aeruginosa*. Two patients from both control and MS had mirror patterns against *Pseudomonas aeruginosa*. All of the patterns persisted up to 2.5M NaSCN with the exception of one of the MS patients where the pattern could not be seen after 1.25M NaSCN.
**Proteus mirabilis.** Two control and two MS samples had mirror patterns against

*Proteus mirabilis.* All of the patterns were visible at 2.5M NaSCN. *Proteus mirabilis*

was chosen as a control organism in its capacity as a classic enteropathogen.

**Escherichia coli.** Three control patients had mirror patterns visible up to 2.5M NaSCN,

but only two of the MS patients, again visible up to 2.5M NaSCN.

Results of antigen blotting are summarised in Table 4.12 for control patients and Table

4.13 for MS patients.

All samples were also blotted onto a membrane impregnated only with blocking

solution, (2% skimmed milk and 1% Tris, both weight/volume dilutions) as a negative

control. One MS patient, MS4, had OCBs that were seen in the serum only. The control

blot was not exposed to NaSCN as the only bands seen were in the serum of a patient in

which no antigen-specific OCBs were found.
Figure 4.25 Mirror pattern and positive response to *A. calcoaceticus*.

Key: A, The typical mirror pattern is represented here by control sample 8, blotted against *Pseudomonas aeruginosa*; B, The strong band (indicated by arrow) in the CSF of MS patient 10, blotted against *Acinetobacter calcoaceticus*. S, serum; C, CSF.

### 4.4.1 Statistical analysis

Fishers exact test showed no significant difference between MS patients and controls for any of the bacteria studied, *Acinetobacter calcoaceticus*, p = 0.2553, *Pseudomonas aeruginosa*, p = 1.000, *Proteus mirabilis*, p = 1.000, *Escherichia coli*, p = 0.3473.
4.5 *Chlamyphilia pneumoniae* in multiple sclerosis

The proposal that *Chlamyphilia pneumoniae* could be an aetiological agent in MS (Sriram *et al*, 1999) prompted an investigation into whether the presence of CSF oligoclonal IgG correlated with the presence of serum IgG raised against *Chlamyphilia pneumoniae*. This was part of a larger study (Furrows *et al*, 2004) designed to look for evidence of current or previous infection in both CSF, using PCR and cell culture, and in serum using ELISA and MIF.

In the 27 control patients, none of who demonstrated intrathecal synthesis of IgG, 2 patients had serum antibodies against *Chlamyphilia pneumoniae*. One had no detectable OCBs in CSF or serum and one had a mirror pattern (identical band pattern in serum and CSF).

Of the 27 control patients 2 were positive on the Western blot for serum IgG against *Chlamyphilia pneumoniae* (7.4%). These were 2 of the 12 patients who were positive on ELISA (44.4%); 2 for IgM only, 8 for IgG only and 2, which were positive for IgM and IgG. Subsequent analysis by MIF confirmed only 2 of these 12 samples to be positive for IgG, two for IgM and one for IgG and IgA. The remaining 7 ELISA positives were negative on MIF. Only samples positive by ELISA were subject to follow-up analysis by MIF. Thus, 5/27 (18.5%) control patients were antibody-positive and 3/27 (11.1%) positive for IgG.

None of the control patients were CSF culture-positive or CSF PCR-positive (Table 4.14).

In the cohort of 19 MS patients, all of who showed intrathecal synthesis of IgG, only one patient, who had no discrete bands of serum IgG, had serum antibodies against *Chlamyphilia pneumoniae*. Seven of the MS patients also showed serum OCBs, but none of these had a specific response to *Chlamyphilia pneumoniae* in the WB.
Table 4.14 Summary of analyses for controls in C. pneumoniae in MS study.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>IEF pattern of total IgG in serum and CSF</th>
<th>Serum Western Blot</th>
<th>ELISA serology</th>
<th>MIF serology</th>
<th>CSF culture</th>
<th>CSF PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>- Positive IgG</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>4</td>
<td>- Negative IgG, IgA</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>- Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>6</td>
<td>- Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>8</td>
<td>- Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>10</td>
<td>- Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>11</td>
<td>- Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
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</tr>
<tr>
<td>14</td>
<td>- Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
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<td>Negative</td>
</tr>
<tr>
<td>16</td>
<td>- Negative</td>
<td>IgG</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>17</td>
<td>- Negative</td>
<td>IgG</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>18</td>
<td>- Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>21</td>
<td>- Negative</td>
<td>IgM</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>22</td>
<td>- Negative</td>
<td>IgG, IgM</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>23</td>
<td>- Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>24</td>
<td>+ Negative</td>
<td>IgG, IgM</td>
<td>IgM</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>26</td>
<td>- Negative</td>
<td>IgG</td>
<td>IgM</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>27</td>
<td>- Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>28</td>
<td>- Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
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<tr>
<td>30</td>
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<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>36</td>
<td>- Negative</td>
<td>IgG</td>
<td>IgG</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>37</td>
<td>- Negative</td>
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<td>Negative</td>
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<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>38</td>
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<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>62</td>
<td>- Negative</td>
<td>IgM</td>
<td>IgM</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>64</td>
<td>- Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>65</td>
<td>- Negative</td>
<td>IgG</td>
<td>Negative</td>
<td>Negative</td>
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<td>68</td>
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<td>69</td>
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</tr>
</tbody>
</table>

For interpretation of IEF patterns, see Table 1.1. Oligoclonal band pattern reporting in Neuroimmunology laboratory, Institute of Neurology.
Table 4.15 Summary of analyses for MS cohort of *C. pneumoniae* in MS study.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>IEF pattern of total IgG in serum and CSF</th>
<th>Serum Western Blot</th>
<th>ELISA serology</th>
<th>MIF serology</th>
<th>CSF culture</th>
<th>CSF PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>&gt;</td>
<td>Negative</td>
<td>IgM</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>Negative</td>
<td>IgG</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>13</td>
<td>*</td>
<td>Negative</td>
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<td>Negative</td>
<td>Negative</td>
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</tr>
<tr>
<td>15</td>
<td>+</td>
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<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>19</td>
<td>&gt;</td>
<td>Negative</td>
<td>IgG, IgM</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>25</td>
<td>+</td>
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<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>29</td>
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<td>IgG</td>
<td>IgA</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>31</td>
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<td>Negative</td>
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<td>Negative</td>
<td>Negative</td>
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</tr>
<tr>
<td>34</td>
<td>-</td>
<td>Negative</td>
<td>IgG</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>35</td>
<td>&gt;</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>39</td>
<td>&gt;</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
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</tr>
<tr>
<td>52</td>
<td>&gt;</td>
<td>Negative</td>
<td>IgG</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>61</td>
<td>+</td>
<td>Positive</td>
<td>IgG, IgM</td>
<td>IgM</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>63</td>
<td>+</td>
<td>Negative</td>
<td>IgG</td>
<td>IgM</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>66</td>
<td>+</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>67</td>
<td>+</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
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<td>+</td>
<td>Negative</td>
<td>IgG, IgM</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

For interpretation of IEF patterns, see Table 1.1 Oligoclonal band pattern reporting in Neuroimmunology laboratory, Institute of Neurology.

Of the 19 MS patients only one was positive on the Western blot for serum IgG against *Chlamydophila pneumoniae* (5.3%). This patient had had confirmed *Chlamydophila pneumoniae* infection 6 years previously. This was one of the 9 patients who were positive on ELISA (47.4%); one for IgM only, 6 for IgG only and two of which were positive for IgM and IgG. Subsequent analysis by MIF confirmed only 1 of these 9 samples to be positive for IgG, one for IgA and two for IgM. The remaining three ELISA positives were negative on MIF. One patient was positive on ELISA for IgM only, which was negative by MIF. Only samples positive by ELISA were subject to follow-up analysis by MIF. Thus, 4/19 (21.1%) MS patients were antibody-positive of which 1/9 (11.1%) was IgG positive.
Figure 4.26 Two patient sera with serum antibody response to *C. pneumoniae*.

Key: A and B, respective traces for patients 38 and 2 against *C. pneumoniae*; C and D, respective traces for patients 38 and 2 against control antigen. The control patient, 61, was also positive but weakly so and could not be reliably digitised.

One of the MS patients was positive for CSF culture and a case report was published by Furrows *et al* (2004) but none was CSF PCR-positive (Table 4.15).

4.6 Turbidimetric assay for precipitation by sodium thiocyanate

The purpose of this assay was to investigate the possibility that 5M NaSCN caused precipitation of protein. It was first observed during the *Acinetobacter calcoaceticus* in MS study that traces on the immunoblots that had been incubated with 5M NaSCN and visualised using ECL showed interference that rendered the traces uninterpretable.
(Figure 4.27). Similar effects were seen with colorimetric development using ethylaminocarbazole when studying the difference between a band in the serum and CSF of an SSPE patient (Figure 4.28) and subsequently during the attempt to demonstrate affinity maturation in an HSE patient.

![Figure 4.27 Uninterpretable trace at 5M NaSCN on ECL development.](image)

Uninterpretable trace at 5M NaSCN on chemiluminescent development of *E. coli* blot.

![Figure 4.28 Uninterpretable trace at 5M NaSCN on colorimetric development.](image)

Uninterpretable trace at 5M NaSCN on colorimetric development of SSPE blot.

The results show no trend when NaSCN was added to diluted serum or saline (Tables 4.16 and 4.17). This would suggest that the addition of NaSCN did not cause non-
specific precipitation of protein. The highest response with 5M NaSCN was seen when 100 μl was added to serum diluted 1/100 (Table 4.16). Likewise, no trends were observed with the addition of 10M NaSCN, which was used to compensate for possible dilution effects of adding 5M NaSCN to diluted serum (Table 4.17). The highest responses were seen when 100 μl of 10M NaSCN was added to the dilutions but the largest change occurred when 100 μl of 10M NaSCN was added to saline.

<table>
<thead>
<tr>
<th>Serum dilution</th>
<th>Δ OD_{340} 5μl</th>
<th>Δ OD_{340} 10μl</th>
<th>Δ OD_{340} 25μl</th>
<th>Δ OD_{340} 50μl</th>
<th>Δ OD_{340} 100μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/50</td>
<td>-0.003</td>
<td>0.008</td>
<td>0.003</td>
<td>-0.001</td>
<td>0.012</td>
</tr>
<tr>
<td>1/100</td>
<td>-0.004</td>
<td>0.013</td>
<td>0.002</td>
<td>0.009</td>
<td>0.030</td>
</tr>
<tr>
<td>1/200</td>
<td>0.012</td>
<td>0.001</td>
<td>0.002</td>
<td>0.004</td>
<td>0.019</td>
</tr>
<tr>
<td>1/400</td>
<td>0.008</td>
<td>0.013</td>
<td>0.015</td>
<td>0.024</td>
<td>0.012</td>
</tr>
<tr>
<td>1/800</td>
<td>0.010</td>
<td>0.009</td>
<td>-0.007</td>
<td>0.019</td>
<td>0.010</td>
</tr>
<tr>
<td>1/1600</td>
<td>0.006</td>
<td>0.016</td>
<td>0.006</td>
<td>0.011</td>
<td>0.012</td>
</tr>
<tr>
<td>1/3200</td>
<td>-0.002</td>
<td>0.018</td>
<td>0.014</td>
<td>0.025</td>
<td>0.012</td>
</tr>
<tr>
<td>Saline</td>
<td>0.012</td>
<td>0.004</td>
<td>-0.009</td>
<td>-0.002</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Data is shown as the difference in absorbance at 340 nm, (Δ OD_{340}),

(OD_{340} sample + NaSCN) − (OD_{340} sample + saline)

where the test represents the addition of NaSCN and the control, the addition of saline.
Table 4.17 Effect of 10M NaSCN on goat anti-HSA.

<table>
<thead>
<tr>
<th>Serum dilution</th>
<th>Δ OD_{340} 5μl</th>
<th>Δ OD_{340} 10μl</th>
<th>Δ OD_{340} 25μl</th>
<th>Δ OD_{340} 50μl</th>
<th>Δ OD_{340} 100μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/50</td>
<td>-0.005</td>
<td>0.008</td>
<td>-0.012</td>
<td>0.011</td>
<td>0.033</td>
</tr>
<tr>
<td>1/100</td>
<td>-0.004</td>
<td>0.018</td>
<td>-0.010</td>
<td>0.014</td>
<td>0.037</td>
</tr>
<tr>
<td>1/200</td>
<td>0.003</td>
<td>0.012</td>
<td>-0.017</td>
<td>0.018</td>
<td>0.032</td>
</tr>
<tr>
<td>1/400</td>
<td>0.000</td>
<td>0.005</td>
<td>-0.012</td>
<td>0.011</td>
<td>0.030</td>
</tr>
<tr>
<td>1/800</td>
<td>0.000</td>
<td>0.011</td>
<td>-0.016</td>
<td>0.013</td>
<td>0.041</td>
</tr>
<tr>
<td>1/1600</td>
<td>-0.006</td>
<td>0.017</td>
<td>-0.012</td>
<td>0.012</td>
<td>0.026</td>
</tr>
<tr>
<td>1/3200</td>
<td>-0.002</td>
<td>0.003</td>
<td>-0.007</td>
<td>0.023</td>
<td>0.035</td>
</tr>
<tr>
<td>Saline</td>
<td>0.005</td>
<td>0.012</td>
<td>-0.030</td>
<td>0.005</td>
<td>0.072</td>
</tr>
</tbody>
</table>

Data is shown as the difference in absorbance at 340 nm, (Δ OD_{340}),

(OD_{340} sample + NaSCN) − (OD_{340} sample + saline)

where the test represents the addition of NaSCN and the control, the addition of saline.

4.7 Rocket electrophoresis

Rocket electrophoresis was used to estimate serum and CSF IgG of paired samples so that the ratios could be determined and hence the appropriate dilutions for IEF.

Wherever this correction was applied it is referred to in the appropriate sections.
5.0 Discussion

5.1 Model Method

The aim of this method was to demonstrate that relative affinity could be quantified using IEF to separate the clones of IgG, affinity-mediated immunoblotting to capture them and NaSCN to probe their relative affinities. Following digitisation of the blot, peak areas in pixel density could be calculated and compared and so this is a potential method for studying both clonality and affinity of sample IgG to a known antigen, or source of antigen. A statistically significant difference was shown between pixel density at 5M NaSCN and pixel density at 0, 1.25 and 2.5M NaSCN although there was no demonstrable difference between 0, 1.25 and 2.5M. However, trend analysis did show a significant trend from 0M to 5M. This is best explained by showing that the polyclonal background is stripped from the membrane faster than the band of high affinity antigen-specific IgG, thus the pixel density of the target band represents a higher proportion of the total pixel density under the curve as the molarity of the NaSCN increases. To emphasise that it is antigen-specific high-affinity antibody pixel densities were compared, from the densitogram, of the target band with an area of polyclonal IgG (see Table 4.3, Figure 4.6) for blots 3 and 18. This showed that while there is little difference at 0, 1.25 and 2.5M NaSCN, at 5M NaSCN the ratio rises markedly, confirming that the polyclonal background is diminished in proportion to the bands of antigen-specific IgG, which are of higher affinity.

Ultrabind was chosen as the membrane because of its ability to bind protein covalently. The matrix of the membrane is polyethersulphone and it contains aldehyde groups, which covalently bind protein molecules through a primary amine group. Covalent binding was considered to reduce the possibility of NaSCN stripping antigen from the membrane as may happen with other membranes such as nitrocellulose or PVDF, where
the antigen binds by non-covalent interactions. This makes them vulnerable to a chaotrope such as NaSCN, which disrupts non-covalent binding, and so at higher concentrations would increase antigen displacement from the membrane alongside antigen antibody disruption. Thus, the calculations of relative affinity would be artefactually lowered. Comparable blots on nitrocellulose showed areas that had little or no colour suggesting that albumin either failed to bind during coating or was removed during the assay. Also, background staining with ethylaminocarbazole was higher on nitrocellulose than Ultrabind, resulting in the dried nitrocellulose being noticeably brown as opposed to the faint pink or white found with Ultrabind. The manufacturers of Ultrabind recommend colorimetric detection with chloronaphthol rather than ethylaminocarbazole. Although this does result in even lower background, work with biological systems suggests that some sensitivity would be lost.

Normal practice in this laboratory is to block membranes using 2% w/v dried skimmed milk in 0.9% saline but initial experiments using this block solution showed it to be incapable of blocking the membrane fully. It was then attempted to block the membrane with 1% Tris in 0.9% saline but this also failed to block the membrane completely. It was found that Ultrabind required blocking in 2% w/v dried skimmed milk and 1% w/v Tris in 0.9% saline. When focused samples of anti-albumin IgG were blotted onto a milk-coated or Tris-coated membrane, faint IEF patterns could be seen at all molarities of thiocyanate. When dried skimmed milk and Tris were combined, no patterns could be seen suggesting that milk proteins or Tris alone were not capable of saturating the aldehyde groups of the membrane that covalently bind proteins. The pore size of nitrocellulose and Ultrabind are the same, 0.45 μm, so although milk is probably capable of blocking the polyethersulphone matrix of the membrane, as it does in nitrocellulose, Tris, with its primary amine group, is probably more efficient at blocking the aldehyde groups.
Antiserum was diluted at 1/1000 to avoid problems with saturation whereby bands on the developed membrane are visible on the reverse of the membrane. This is thought to be lower affinity antibody that passes through the membrane quickly (Luxton and Thompson, 1989). Saturation can also manifest as heavy, non-discrete staining on the front (developed) side of the membrane. This was not acceptable for the process of quantifying the bands which needed linearity. Double-dilution of the anti-human albumin down to 1/16000, gave progressively fainter patterns, both with respect to dilution at the same molarity of NaSCN and at the same dilution over different molarities of NaSCN. When the antiserum was diluted to 1/2000 throughout a single experiment, it was found that the traces lacked consistency.

In accordance with previous work (Pullen et al, 1986; Luxton and Thompson, 1990) and previous studies in this laboratory, NaSCN was used as it had been shown to be an efficient chaotrope. Initially, 10M NaSCN was used as the highest concentration but it was found that it stripped almost the entire antibody from the membrane. As a result 5M NaSCN was adopted as the highest concentration, which was in line with previously published work, both with ELISA and immunoblots. Given that the commercial antiserum was rich in high affinity antibody to human albumin, incubation with NaSCN was for 30 minutes. Observations in this laboratory suggest that this should be reduced when studying biological systems as the antigen source may not be pure nor may the antibody response be as strong.

Initially, gels were run according to the method of Keir et al (1990) but subsequently gels were focused at starting conditions of 800 V, 150 mA and 20 W for 900 volt hours. This was because a number of traces showed a pronounced “bend” when developed which made them unsuitable for quantitative analysis. To counter this problem, possibly caused by excess heat from the electrophoresis the voltage was reduced (and hence, the heat) and thus extended the run time whilst maintaining the volt hours. A domestic fan
was directed onto the tank whilst running to increase heat removal from the system. To ensure that the current was equally applied across the width of the gel the electrodes were cleaned with 1:1 methanol: water after each run to remove any deposits which may have built up during the run and interfered with conduction. The electrodes were also cleaned monthly with a 1% Decon (Decon Laboratories Ltd.) solution.

The first antibody, RAG, was applied at a 1/2500 dilution in 0.2% skimmed milk in saline and second antibody, SAR conjugated to HRP, was applied at 1/5000 dilution, i.e. 10 μl in 25 ml and 5 μl in 25 ml, respectively. Had the membrane been left intact, rather than being divided into four parts, then a total of 40 μl of first antibody would have been used but the dilution volume would have been reduced to 50 ml. Therefore, a slightly lower absolute amount of first antibody was used than in the IEF method for OCBs routinely used in this laboratory, which uses 50 μl. However, the second antibody dilution would have been the equivalent of 20 μl across the whole membrane as opposed to the 50 μl routinely used for IEF. This reflects the more sensitive nature of an assay detecting specific IgG rather than total IgG, which is detected by IEF for OCBs.

Digitised images were saved as greyscale TIFFs because of the universal nature and ease-of-use of these formats. This allowed us to standardise all blot analyses by calibrating with a commercially-produced and freely available standard curve, in the form of a graded step tablet, and to express results in terms of optical (pixel) density, which is an internationally recognised unit of measurement. The only drawback was the need for access to a scanner capable of scanning high resolution images; the software used (Irfanview and ImageJ) are both available as free downloads from the internet, ImageJ being a Java-based program that is platform-independent.

The same batches of commercially available antigen and antiserum were used in all blots to remove two potential sources of variation across all experiments and were deliberately chosen so that the method could be repeated by other workers to verify the
results. Commercial antigen and antiserum should have little batch-to-batch variation with regard to purity and activity, and therefore represent the optimal form of the method; “in-house” or commercial preparations of bacterial and viral antigen will not necessarily be as pure. The purity of the human albumin meant that, compared to bacterial antigen preparations or even commercial viral antigen preparations, a much lower protein load was necessary to sufficiently coat the membrane with antigen. Likewise, the commercial anti-human albumin antiserum was rich in specific high-affinity antibodies to human albumin due to repeated immunisations of the source animal, resulting in strong and consistent patterns when the two were combined. For these reasons, it would be recommended that in such experiments preliminary studies are conducted to determine an appropriate coating capacity and sample dilutions should also be studied to determine an optimum dilution for the level of coating. Preferably, the antigen will be in excess, allowing the capture of the maximum amount of antibody, whilst maintaining a low polyclonal background.

To confirm that the anti-human albumin antiserum did indeed contain high affinity antibodies against human albumin a modified version of the affinity distribution was performed (Luxton and Thompson, 1990) using 0M (saline), 1.25M, 2.5M, 5M and 10M NaSCN. This showed that high affinity antibodies were present at both 5M and were even detectable at 10M NaSCN thus providing confirmation by another method of their presence.

5.2 Comparison of band in serum and cerebrospinal fluid

A paired sample of serum and CSF was taken from a patient referred to the NHNN with a clinical diagnosis of SSPE with the intention of demonstrating that the same clone in the CSF and serum was significantly stronger in CSF. Although weak bands could be seen by eye they were not always detectable on the densitogram or the scanned image. As the concentration of NaSCN increased polyclonal background IgG was lost more
quickly than antigen-specific IgG thus the samples were more dilute for exposure to 0M NaSCN so as to maximise the signal from the serum trace whilst avoiding saturated areas of the membrane. This could not be done wholly successfully and the representative band scanned did not include the whole trace. Nevertheless, the pixel density of the area scanned from each trace was identical, as ImageJ allows the same box to be applied to each trace and gives co-ordinates to ensure correct positioning. Routine IEF of the paired samples revealed the patient to have a “greater than” (\(\gg\)) pattern that had many more, and stronger bands, in the CSF than in the serum. However, affinity-mediated immunoblot revealed a mirror pattern with regard to measles-specific IgG. The ratio between the serum and CSF bands remained consistent at each molarity for at least two of the three replicates, indicating that the measles-specific serum IgG was of largely intrathecal origin, as the ratio was maintained. The mirror pattern is almost certainly a reflection of the chronic condition of the patient in that intrathecal IgG produced against measles slowly leaches into the systemic circulation. That the patient sample was taken at least two months after the first reported symptoms is consistent with this conclusion. It may be that the assay is sufficiently sensitive to detect the leak from CSF into serum, however, there will also be some production of systemic antibody by the Peyer’s patches in response to chronic measles infection. Another factor that would affect the different patterns is the blotting techniques used in the two assays. In routine IEF the background of polyclonal IgG is greater as total IgG is being detected; in affinity blotting, detection is biased towards antigen-specific IgG, therefore bands that are “swamped” in IEF can become visible in affinity blotting. A 1:400 dilution of serum, as in IEF, yields a concentration of 23 mg/l IgG; in affinity blotting the serum dilutions gave 4.4 mg/l at 0M NaSCN and 8.8 mg/l at 1.25 and 2.5M NaSCN. That bands became visible in affinity blotting at lower concentrations suggests that the technique, as would be expected, is more sensitive than routine IEF at detecting specific
antibody. This, of course, is dependent on the amount of specific IgG that is actually present in the sample. Moreover, with statistically significant differences between serum and CSF replicates at each molarity, it suggests that the method can also be capable of distinguishing intrathecally-produced antigen-specific IgG. This agrees with the original proposal of Moyle and Thompson (1984), that the technique is capable of detecting low levels of antigen-specific antibody, such as would be found in early disease. The sample had a normal CSF total protein of 0.36 g/l, usual in SSPE, indicating that blood-brain barrier function was intact and that there was minimal passive transfer of systemic IgG into the CNS, including antigen-specific IgG of systemic origin.

It could be argued that the polyclonal background in CSF is derived from plasma and that including it when calculating peak areas of specific IgG as a percentage weakens the test, when comparing serum to CSF. To counter this, simply measuring the peak height ratio of serum to CSF could be seen as more appropriate. However, by looking at antigen-specific antibody and diluting serum and CSF to the same concentration of IgG, background IgG was considerably minimised, which therefore reduced the background effect of polyclonal IgG.
5.3 Affinity maturation in Herpes simplex encephalitis

The diagnosis of HSE (Type 1 Herpes virus) was initially made on the basis of the PCR for viral DNA on the first CSF sample being positive. Standard clinical practice is to prescribe acyclovir when encephalitis is suspected, even though it may not be caused by herpes, and so the patient was on acyclovir from admission. There is only a small "window of opportunity" in which PCR will produce positive results (Sindic et al, 2003) and that, combined with the acyclovir therapy, is probably responsible for the subsequent negative results on PCR in the CSF. Another strong indication of the cause of the encephalitis being of viral origin, rather than any other, is the white cell count. Normal range CSF white cell count is less than 5 per mm$^3$; the first sample contained 200 lymphocytes, the second 190 white cells, which were predominantly lymphocytes and the last sample contained 36 white cells, again predominantly lymphocytes. Lymphocytes are usually found in viral infections whereas polymorphonuclear neutrophils (PMNs) are usually raised in bacterial meningitis. The reduction in the numbers of lymphocytes over the sixteen days that the samples span is probably a reflection of the resolution of the infection by a combination of the immune response and the acyclovir therapy. Along with the positive PCR, the white cell counts are indicative of CNS infection by a viral agent. Outside of routine diagnostic testing, rocket electrophoresis for total IgG in both serum and CSF was also performed. This showed that the total IgG in CSF one day after admission was 260 mg/l which rose to 845 mg/l after six days and declined slightly to 696 mg/l after sixteen days. Normal CSF IgG is approximately 25 mg/l, thus levels were 10-fold, 34-fold and 28-fold higher, respectively, on days 1, 6 and 16 after admission. Serum levels rose from 7585 mg/l to 16367 mg/l over the same time. This rapid increase in CSF IgG strongly suggests that the IgG produced is intrathecal, rather than systemic which nevertheless doubled, probably due to accumulation of intrathecal IgG in the serum and some systemic IgG
production from the spleen against virus particles and fragments entering the systemic circulation from the CSF. Thus, it was reasonably certain that the diagnosis of HSE was correct.

The total area under the curve, as measured from the densitograms, rose from day one through to day three, in both serum and CSF, without exception. The total pixel density fell as the molarity of the NaSCN rose in both serum and CSF. However, only at 5M NaSCN did the total pixel density in CSF drop below any of the serum total pixel density levels, suggesting that the CNS production of high-affinity antigen-specific IgG was in higher proportion than in the serum. As the assay was specifically designed to capture antigen-specific antibody, this shows that in both serum and CSF, antigen-specific high-affinity IgG was being produced whilst low-affinity IgG was being progressively removed by stronger NaSCN. Although the amount of high-affinity IgG rose in CSF the total IgG dropped slightly between days 6 and 15. This was interpreted as the maturing of the immune response, producing more high-affinity IgG, but of restricted clonality, a hallmark of affinity maturation. In serum, the total IgG and antigen-specific IgG both rose between days 6 and 15, albeit less dramatically than in CSF. As the seat of the original infection was in the CNS, the systemic response to the herpes virus would start later as viral particles and fragments cross the BBB into the systemic circulation. Previous work has suggested that B cell clones may also be recruited in the periphery to reinforce the initial intrathecal response (Davies, 1988).

Ideally, a paired sample from the convalescent phase would have been taken, allowing investigation into the clonal range and affinity of specific IgG post infection in serum and CSF, but in clinical terms this is not necessary as the illness is resolved or resolving. It would be predicted that the total and specific IgG would have returned to normal levels and the number of clones would be fewer. However, those clones remaining would still be of high affinity.
The experiment was repeated at two normalised concentrations of total IgG; 52 mg/l and 104 mg/l. Although it was apparent that much of the trace at 104 mg/l would be saturated it allowed the investigator to see bands that were far more subtle at higher dilutions. More bands were seen in the serum and CSF of the first sample at 104 mg/l than in the 26 mg/l sample but at neither dilution did these bands persist beyond 0M NaSCN. Both the second and third samples, diluted at 26 mg/l and 104 mg/l, showed far stronger bands at 0M NaSCN which were still clearly visible at 5M NaSCN. This was interpreted as showing that the early sample contained less specific IgG than the latter two, which was indicated by rocket electrophoresis for total IgG, and that the specific antibody was not of sufficient affinity to resist 5M NaSCN. The later two samples, which had approximately three times the total IgG, also contained specific IgG of high affinity for herpes virus. The presence of specific IgG of high affinity, it was concluded, demonstrated that affinity maturation with regard to herpes virus had occurred from the time of the first sample to the time of the second sample.

Interestingly, the experiment at 52 mg/l was the last one performed on these paired samples. The trace itself had run shorter than the 26 mg/l and 104 mg/l traces, making it somewhat harder to see the bands present. Tellingly, this was the only experiment where the "interference" effect at 5M NaSCN was seen. This would suggest that the highest dilution, 26 mg/l, was most reliable as this equates to normal CSF levels of total IgG, which do not saturate the antigen-coated membrane.

The patient had a greatly raised total protein within the CSF indicating blood-brain barrier damage, suggesting that the infection was already established at admission. Poor barrier function may allow some passive movement of IgG between the systemic circulation and the CNS. As the samples were diluted to parity with respect to IgG concentration, rather than assuming that serum could be diluted 1/400 to normalise background total IgG, this could have enhanced the appearance of bands in the serum.
5.4 *Acineobacter calcoaceticus* in MS

The previous study by Hughes *et al* (2001) looked at the serum immune response to *Acinetobacter calcoaceticus, Pseudomonas aeruginosa, MBP* and neurofilaments in MS, cerebrovascular accident and healthy controls to calculate an index by which MS and non-MS could be distinguished. Using a semi-quantitative ELISA they showed significantly higher levels of IgG against *Acinetobacter calcoaceticus* and *Pseudomonas aeruginosa* in serum from MS patients.

As MS is a disease characterised by the intrathecal synthesis of OCBs of IgG, it was necessary to determine whether or not any IgG detected was of systemic or intrathecal origin. For this paired CSF and serum that were taken contemporaneously were compared. With just one possible exception, any patient, either MS or control, who showed OCBs in this study, had a mirror pattern implying the IgG was of systemic origin. Although this would not rule out an autoimmune mechanism, whereby infection of the periphery could lead to activation of autoreactive B cells and production of cross-reactive antibodies which could then enter the CNS, the results of these assays do not support a role. Indeed, there was a higher incidence of the mirror patterns in the control patients than the MS patients for *Acinetobacter calcoaceticus* and equal occurrence of *Pseudomonas aeruginosa*, supporting the lack of association.

The possible exception, mentioned above, was the MS patient blotted against *Acinetobacter calcoaceticus*. Although the same bands were present in both serum and CSF, they were of a lesser intensity in the serum, which could suggest they were of intrathecal origin. However, the method of visualisation, ECL, does not show a linear response and, as sera were routinely diluted 1 in 400 on the basis of normal barrier function rather than IgG concentrations being standardised, it was impossible to determine whether this was a reflection of the respective concentrations of total IgG.
Attempts to visualise the results using colorimetric detection were unsuccessful due to the relative insensitivity compared to ECL.

Another characteristic of MS is the polyspecific low affinities of the oligoclonal IgG intrathecally produced (Luxton et al., 1995). In this study blots were therefore incubated with varying concentrations of the chaotropic agent, NaSCN, to determine the affinity of any IgG detected. As no evidence of intrathecal synthesis was found, with the possible exception previously discussed, the question of affinity became academic. However, it was found that the patterns persisted in both CSF and serum up to the same concentration of NaSCN, usually 2.5M. In some instances, the blots that were incubated in 5M NaSCN were uninterpretable owing to interference possibly caused by the NaSCN itself (Bouvet et al., 2001a). The similarity of serum and CSF band patterns suggests that the IgG in the CSF is a “leak” from the serum, therefore having the same affinity, and does not originate intrathecally.

One of the MS patients, MS4, showed serum, but not CSF, oligoclonal IgG against milk proteins on the control blot. The lack of corresponding bands in the CSF is probably due to their lower concentration as CSF samples were run undiluted.

The band patterns observed in these experiments bore no obvious relation to those seen on the original IEF. The patterns seen were often restricted to a small area of the overall trace whereas on the original IEF the bands were distributed widely across the pH gradient. What may have been detected was a recent “challenge” to the immune system following an encounter with the organism or even the immune memory of a previous encounter. It should be noted, however, that the encephalitogenic sequence of MBP is recognised by T cell receptors and not necessarily B cells (Mazza et al., 2002) thus there may be no intrathecal antibody response. Also, the work by O’Connor et al. (2003), which could only find low affinity IgG against MBP in MS serum and CSF, suggested that MBP was not a major target of autoantibodies in MS. However, this does not
discount the possibility that antibodies with higher affinity are produced and bound to myelin within CNS tissue. Within the confines of this study the results seem to indicate that exposure to *Acinetobacter calcoaceticus* or *Pseudomonas aeruginosa* does not occur at a higher frequency in MS patients compared to those with other neurological diseases. It is therefore questionable whether infection with either of the bacteria may be involved in a pathogenic autoimmune mechanism leading to MS.

The theory of *Acinetobacter sp.* causing neurological disease by molecular mimicry of MBP was first proposed by Ebringer et al (1997) as a possible cause of BSE, following accidental contamination of cattle foodstuffs with bowel bacteria. Further work by the same group (Tiwana et al, 1999) found raised serum antibodies to MBP and *Acinetobacter sp.* in BSE-affected cattle though the significance of this was questioned (Nielsen et al, 2002). On the basis of the sequence homology between *Acinetobacter sp.* and MBP and the presence of MBP-specific T cell clones in MS patients (Wucherpfennig and Strominger, 1995) *Acinetobacter sp.* were proposed as potential aetiological agents in MS. However, the absence of common clinical findings, such as demyelination and intrathecal synthesis of IgG in MS, would suggest that there are few parallels to be drawn between the two pathologies. The final argument would be the level of homology. Usually a sequence of at least eight amino acids is required to be homologous for there to be a likelihood of the two antigens containing the same target. None of the proposed sequences has more than five homologous amino acids and none is in a continuous sequence.
5.5 *Chlamyphila pneumoniae* in MS

Following Sriram’s original study (1999), a number of further studies were undertaken to investigate the proposed link between MS and *Chlamyphila pneumoniae*. None of these studies, however, could confirm Sriram’s findings. In order to investigate the association shown by Sriram *et al* (1999), similar methodology was used, concentrating on CSF culture, CSF PCR and serology. Although the culture of *Chlamyphila pneumoniae* from the CSF of an MS patient is unique in the United Kingdom, PCR from this, and all other samples, proved to be negative. Internal controls in the PCR proved that 90% of the assays were capable of detecting 125 genomes ml/CSF. However, although all samples were reported as negative this included those with negative internal controls when the sensitivity of the assay was unknown. The culture-positive sample had a negative internal control, which may explain the negative PCR finding. The serology studies on this one patient confirmed previous infection, which supported the finding of serum IgG against *Chlamyphila pneumoniae* found in the Western blotting. One of the two control samples that were positive on the Western blot was negative for OCBs in serum by IEF, which was detected colorimetrically, whereas the Western blotting was detected by chemiluminescence, a more sensitive method. Thus, it may be that the Western blotting was simply detecting circulating antibody from previous exposure to *Chlamyphila pneumoniae*. This would be supported by the result from the MS patient.

Although all the Western blot positive patients were positive for IgG by ELISA serology, only the positive MS patient was also positive by MIF. Furrows *et al* (2004) found four MS patients and six controls to be positive by MIF suggesting that WB is not sufficiently sensitive to replace MIF, the current “gold standard” for serodiagnosis of *Chlamyphila pneumoniae* infection despite attempts to use recombinant chlamydial proteins rather than cell line homogenates (Pucci *et al*, 2000).
In summary, while these results showed that *Chlamydophila pneumoniae* could be cultured from the CSF in 1 of 19 patients with MS, serology did not demonstrate a difference in exposure between the two groups, and PCR was negative in all cases. It was therefore concluded that within this cohort there was no evidence of systemic infection with *Chlamydophila pneumoniae* being either a cause of or having any association with the pathogenesis of MS.

However, the recent Nurses' Health Study (Munger *et al.*, 2003) suggests there may be a positive association between *Chlamydophila pneumoniae* infection and MS, particularly progressive MS. There is therefore a need for well-designed clinical and epidemiological studies to produce more direct evidence of a role for *Chlamydophila pneumoniae* infection in MS.
5.6 Interference studies with sodium thiocyanate

In a number of experiments an effect was observed on focused samples after exposure to 5M NaSCN. The effect was manifested as excessive homogenous colour development during visualisation along the length and width of the sample trace, although not beyond it, which obscured or replaced the expected patterns. This suggested that the effect must involve the antibodies in the experiments rather than the antigens or blocking proteins, as the latter were distributed across the entire membrane, as was the colour reagent at the end of the assay. The effect was observed with ECL and colorimetric development, with both ethylaminocarbazole and chloronaphthol, which ruled out the possibility that the effect was an artefact of any particular developing reagent and it occurred with a number of different samples, suggesting that the effect was independent of the samples focused. The effect was not seen on any of the blots run to generate data for the model method.

It was hypothesised that the effect may have been due to precipitation of protein by 5M NaSCN and set up an ELISA to investigate this. Fifty μl of goat anti-human albumin, double-diluted from 1/50 down to 1/3200, with a saline control, were put in an ELISA plate and immediately mixed with either 5, 10, 25, 50 or 100 μl 5M or 10M NaSCN. The samples were diluted in saline, reducing the amount of adsorption of antibody to the plate, which normally requires alkaline pH. Any protein precipitation would have been most marked at the highest concentrations of antibody and highest volumes of NaSCN but there was no discernible trend with either parameter. Indeed, the biggest effect was observed with saline and 10M NaSCN which implies that any changes in turbidity may be due to the viscous nature of concentrated NaSCN. From this it was concluded that the interference effect was not due to protein precipitation. Although previous studies had recognised the phenomenon none had identified it on IEF followed by affinity-mediated immunoblotting. Gray and Shaw (1993) noted the effect on affinity
purified monoclonal human IgM which McMahon and Kennedy (2000), looking at affinity purified murine monoclonal IgM, thought to be an artifact of the affinity purification of the antibody solution. However, as the samples used were not affinity purified it was concluded that although the effect may be mediated by affinity purification it is not an exclusive effect of it. Bouvet et al (2001a) were more comprehensive in their investigations, finding that the effect varied with antibody exposed to the chaotrope before being introduced to the assay, and that it was not artefactual since different chaotropes were effective. They also showed that the effect was probably localised to the F(ab) fragment rather than the Fc and was possibly an irreversible conversion rendering the IgG poly-reactive.

Although these studies concentrated on IgG becoming poly- and self-reactive the principle of structural alteration of the IgG molecule remains. It is possible that the 5M NaSCN in these experiments is modifying the polyclonal, or background, IgG that is binding to the antigen on the membrane, rendering it more “visible” to at least one of the detector antibodies. Band patterns can still be seen in some of the affected traces but it would be difficult to apply any sort of reliable interpretation on them. Another observation made was that the effect seemed to increase with the age of the sample. This implies that the in vivo degeneration of the sample antibodies make them more vulnerable to the effects of NaSCN, further suggesting that any alteration is of a chemical and therefore structural nature. Interestingly, the model method studies using commercial goat anti-human albumin did not suffer from the problem of exposure to 5M NaSCN even though they were exposed for 30 minutes and the same serum was used for a number of weeks. Although the effect does not seem to be species-specific with regard to IgM, the same conclusion cannot yet be drawn for IgG as it has only been reported in human IgG. The last experiment with the series of patient samples from the HSE case demonstrated the effect whereas two previous experiments had not. This
implies that the increasing age of the samples, possibly due to some natural *in vitro* degradation, may be a factor in their susceptibility to high strength thiocyanate. The goat anti-human albumin contains 0.2% sodium azide which normally prevents bacterial growth by inhibiting oxidative enzymes. It may be that it also acts as a scavenger for oxygen and prevents *in vitro* oxidation and thus, reduces its susceptibility to alteration by NaSCN.

**Summary**

To summarise: the technique of Eastern blotting allows the objective measurement of differences in four key areas of a blot. Differences can be shown between:

1. Serum and CSF.
2. Oligoclonal and polyclonal IgG.
4. Affinity, by using different molarities of thiocyanate.
6.0 Conclusions

The results from the model method showed that the relative affinity of clones of antigen-specific IgG could be measured by IEF, antigen-blotting and thiocyanate elution, followed by digitisation of the blot and investigation with image-processing software. As part of this study a covalent-binding membrane was successfully used to immobilise antigen, thus reducing the risk of losing antigen at high molarities of thiocyanate. Statistical analysis showed a significant trend for peak area (in pixel density) as a percentage of the total area to increase with molarity of thiocyanate for a high-affinity clone. Thus, it was concluded that this was a valid method for the investigation of affinity of antigen-specific IgG. To demonstrate this further, the ratio of band plus polyclonal to polyclonal areas was compared for both model method blots, the SSPE patient and HSE series blots. These showed the ratio of "band" to "polyclonal" increased at 5M NaSCN on the model method blots but increased across all molarities on the HSE blot, particularly at 5M NaSCN. The SSPE blot only went up to 2.5M NaSCN but nevertheless demonstrated a sharp rise in ratio with increasing molarity of NaSCN.

The method was slightly adapted, in terms of antigen load and length of exposure to thiocyanate, to investigate clinical cases of SSPE and HSE. When serum and CSF IgG concentrations were normalised, antigen-specific IgG bands were shown to be present in the SSPE serum whereas at the standard dilution of 1/400 for serum and neat for CSF they were not. However, the peak area (in pixel density) of a band in CSF in SSPE was significantly higher than that in the serum, indicating that it was of intrathecal origin, especially as the CSF total protein was within normal range. In the HSE series, the serum bands were still present at 1/400 dilution, reflecting the high amounts of intrathecal IgG produced and loss of integrity of the blood:brain barrier. Over time, IgG passes from CSF to serum (and sometimes vice versa), thus identical bands can be
found in serum and CSF even when they originate intrathecally. As samples were
diluted to parity with respect to IgG concentration this enhanced the appearance of
bands in the serum.

The method is therefore capable of discriminating between subtle differences and it can
be used to show differences between IgG affinity of the same band in serum and CSF or
between bands in the same sample, based on resistance to increasing molarity of
NaSCN.

Two groups of researchers had separately suggested that *Acinetobacter sp.* and
*Chlamyphila pneumoniae* were potentially infectious causes of MS. Sriram (1999)
reported significantly higher levels of *Chlamyphila pneumoniae* by culture and PCR
in CSF. The claim for *Chlamyphila pneumoniae* was investigated in collaboration
with another group by a variety of methods; PCR, serology by ELISA and the more
sensitive MIF, CSF culture and Western blotting. A cohort of MS patients were
compared with a control group of other neurological disorders but no difference
between the two groups was found, leading to the conclusion that infection with
*Chlamyphila pneumoniae* did not lead to the development of MS.

*Acinetobacter sp.* were proposed as causative agents of MS due to a sequence similarity
between one of the bacterial enzymes and MBP. Antibodies raised against the
*Acinetobacter* protein were then thought to cross-react with MBP leading to an immune
response and subsequent demyelination. A cohort of MS patients was compared with a
cohort of other neurological diseases by antigen-blotting of paired serum and CSF
samples against *Acinetobacter calcoaceticus*, with *Pseudomonas aeruginosa*, *Proteus
mirabilis* and *Escherichia coli* as control organisms. Again, no difference was found
between MS and control cohorts and it was concluded that *Acinetobacter calcoaceticus*
was not implicated in the pathogenesis of MS.
As part of the experiments a phenomenon was discovered whereby traces exposed to 5M NaSCN showed interference, which manifested as a "smearing" effect when the blot was visualised. Observations made in this study suggested that the effect was not artefactual, as previous researchers had thought, but a genuine effect mediated by high strength thiocyanate. It did not appear when using commercial goat serum over many weeks, which contains sodium azide as a preservative, but patient samples suffered, particularly when stored for longer. Thus, it was concluded that \textit{in vitro} processes in cold-stored serum might render the immunoglobulin more susceptible to modification by thiocyanate.
7.0 Future work

An interesting and unexpected finding was the interference effect of 5M NaSCN. This phenomenon needs to be further investigated by studying the effects of age, storage and addition of sodium azide to commercial and patient sera to see whether the effect of 5M NaSCN can be modified. It does not appear to be an effect of affinity purification, as one previous study concluded, but would be helpful to understand some of the causes and if possible find a means of prevention, or at least a timescale in which this happens. It would also be of interest to study the ratio between bands of IgG in paired samples of CSF and serum. Given that there is approximately 10000 times more IgG in the serum and Tourtellotte’s argument (1970) that there are insufficient lymphocytes in the CNS to generate enough IgG to appear in the serum this may allow the deduction of whether the B cell clones producing the IgG remain in the CNS, the presence of IgG in the serum being merely an “overspill”, or whether some of the B cell clones are returning to the systemic circulation and producing IgG independently.

A recent study (Cepok et al, 2005) has suggested that EBV may be important in the pathogenesis of MS. Some of their conclusions were based on IEF followed by blotting onto antigen-coated membrane which showed an IgG response to EBV proteins in MS patients. This part of their study should be repeated incorporating incubation with NaSCN to assess the relative affinities of any antigen-specific IgG found.
References


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Appendices

Appendix i. Peptides referred to in *Acinetobacter calcoaceticus* in MS study.


<table>
<thead>
<tr>
<th>Peptide</th>
<th>Source</th>
<th>Amino acid sequence</th>
<th>Amino acid position</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Human</td>
<td>GLSLRSFWSWGAEGQR</td>
<td>110-124</td>
<td>MBP</td>
</tr>
<tr>
<td>2</td>
<td>A. calcoaceticus</td>
<td>QNFISRFAWGEVNSR</td>
<td>38-52</td>
<td>4-CMLD</td>
</tr>
<tr>
<td>3</td>
<td>P. aeruginosa</td>
<td>QEMITRHAWGDIWTR</td>
<td>38-52</td>
<td>γ-CMLD</td>
</tr>
<tr>
<td>4</td>
<td>Human</td>
<td>HARHGFPLPRHRDTGILDS</td>
<td>23-40</td>
<td>MBP</td>
</tr>
<tr>
<td>5</td>
<td>A. calcoaceticus</td>
<td>FKAAPQARKLRDTGIISV</td>
<td>67-84</td>
<td>Coenzyme pqq synthesis protein b</td>
</tr>
<tr>
<td>6</td>
<td>P. aeruginosa</td>
<td>FAPMQPGRALRDTAIGAI</td>
<td>67-84</td>
<td>Coenzyme pqq synthesis protein b</td>
</tr>
<tr>
<td>7</td>
<td>Human</td>
<td>PFSRNVHLRYRNGKDQ</td>
<td>43-57</td>
<td>MOG</td>
</tr>
<tr>
<td>8</td>
<td>A. calcoaceticus</td>
<td>DSYVFDELYRAGKIE</td>
<td>83-97</td>
<td>3-oxoadipate CoA transferase subunit A</td>
</tr>
<tr>
<td>9</td>
<td>Human</td>
<td>EISFLKKVHEEEIAE</td>
<td>219-233</td>
<td>NFL</td>
</tr>
<tr>
<td>10</td>
<td>A. calcoaceticus</td>
<td>SNMDDKKVKEEKILH</td>
<td>3-17</td>
<td>Regulatory protein</td>
</tr>
<tr>
<td>11</td>
<td>Human</td>
<td>KQLQELDKQNADIS</td>
<td>331-345</td>
<td>NFL</td>
</tr>
<tr>
<td>12</td>
<td>A. calcoaceticus</td>
<td>RALIALEDKSNFIEA</td>
<td>203-217</td>
<td>Protocatechuate e-3,4-dioxygenase</td>
</tr>
<tr>
<td>13</td>
<td>HPV (type 16)</td>
<td>TVIQDGDVMVHTGFA</td>
<td>219-233</td>
<td>Major capsid protein L1</td>
</tr>
</tbody>
</table>

Abbreviations: A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; V, valine; W, tryptophan; Y, tyrosine.
Appendix ii. Names and addresses of suppliers

Amersham plc. Including Hoefer
Amersham Place
Little Chalfont
Buckinghamshire HP7 9NA
United Kingdom

Baxter Diagnostics
Wallingford Road
Compton
Newbury
Berkshire RG20 7QW
United Kingdom

BDH (VWR International Ltd.)
Merck House
Poole
Dorset BH15 1TD
United Kingdom

Becton Dickinson including Plastipak
21 Between Towns Road
Cowley
Oxford OX4 3LY
United Kingdom

BioRad Laboratories Ltd.
BioRad House
Maylands Avenue
Hemel Hempstead
Hertfordshire HP2 7TD
United Kingdom

BMA Products (Cambrex Bioscience)
Biocity Nottingham
Pennyfoot Street
Nottingham NG1 1GF
United Kingdom

DakoCytomation Ltd.
Denmark House
Angel Drove
Ely
Cambridgeshire CB7 4ET
United Kingdom

Decon Laboratories Limited
Conway Street
Hove
East Sussex BN3 3LY
United Kingdom

DiaSorin Ltd.
Charles House
Toutley Rd.
Wokingham,
Berkshire RG41 1QN
United Kingdom

Elkay Laboratory Products Ltd.
4 Marlborough Mews
Crockford Lane
Basingstoke
Hampshire RG24 8NA
United Kingdom

Eppendorf UK Limited
Endurance House
Chivers Way
Histon
Cambridge CB4 9ZR
United Kingdom

Hayman Ltd.
Eastways Park
Witham
Essex CM8 3YE
United Kingdom

GlobePharm
PO Box 89C
Esher
Surry KT10 9NA
United Kingdom

Hollingsworth & Vose Company
112 Washington Street
East Walpole
MA 02032
USA

Invitrogen Ltd including GibcoBRL
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF
United Kingdom

MP Biomedicals
Qbiogene
Wellington House
East Road
Cambridge CB1 1BH
United Kingdom
NIH
9000 Rockville Pike
Bethesda
Maryland 20892
USA

Novagen
Merck Biosciences Ltd.
Padge Road
Beeston
Nottingham NG9 2JR
United Kingdom

Oxoid Limited
Wade Road
Basingstoke
Hampshire RG24 8PW
United Kingdom

Pall Gelman
Havant Street
Portsmouth
Hampshire PO1 3PD
United Kingdom

Perbio Science UK Ltd. (Pierce)
Unit 9
North Nelson Industrial Estate
Cramlington
Northumberland NE231WA
United Kingdom

Perkin Elmer (including Wallac)
Chalfont Road
Seer Green
Beaconsfield
Buckinghamshire HP9 2FX

Quadrattech Ltd.
PO Box 167
Epsom
Surrey KT18 7YL
United Kingdom

Roche Diagnostics Ltd.
Bell Lane
Lewes
East Sussex BN7 1LG
United Kingdom

Serva
AMS Biotechnology (Europe) Ltd.
63B Milton Park
Abingdon
Oxfordshire OX14 4RX
United Kingdom

Sigma-Aldrich Ltd.
Fancy Road
Poole
Dorset BH12 4QH
United Kingdom

Stuart Scientific
Barloworld Scientific Ltd.
Beacon Road
Stone
Staffordshire ST15 0SA
United Kingdom

Vienna University of Technology
Karlsplatz 13
1040 Vienna
Austria
Appendix iii. Protocol for use of Irfanview and ImageJ

**IRFAN**

Open the image to be analysed.
Under the **Image** menu select **Convert to Greyscale**
Under the **File** menu select **Save As**, give the file a name and choose file type **TIF**
(Under **Options** select **TIFF** and tick box for **compression – none**)

**ImageJ**

Under the **Edit/Options/Miscellaneous** menu select **Open Images at 100%**.

Open the image to be analysed

Go to **Analyze/Calibrate** and open the file <calibration.txt>.
Select **Function** Rodbard from dropdown menu and change **Unit** to Pixel Density.
Press **OK** and calibration curve will appear in new window. Shrink down using <-> key in top right corner.
On the toolbar the **Square** icon is already highlighted. Move cursor to top left of where required rectangle is to begin. Holding the left mouse button down draw rectangle where required on the image.
Go to **Analyze/Gels/Select first lane** to keep the rectangle.
Move cursor to interior of rectangle, hold down left mouse button and move rectangle to next trace. Note: outline of rectangle will move and does not remain on previous trace.
Go to **Analyze/Gels/Select next lane**. Repeat until all required traces have been captured.
Go to **Analyze/Gels/Plot lanes** and densitograms will appear in new window.

**Straight line** tool is now highlighted in toolbar.
Move cursor onto densitogram and delineate peak by holding down left mouse button to draw lines.
Continue through all densitograms until all required peaks have been identified.

Select **Magic Wand** from toolbar. Click once with mouse in each area to be measured. Results appear in new window.
Click <x> in top right corner of plots to get options on saving the plots. Note: they will be saved with any lines drawn on them. To save results go under **File/Save as option within** Results box not on main ImageJ toolbar. Note: results files are simply a list of numbers. It is advisable to make notes on which results go with which peaks as they are calculated.

Both Plots and Results files can be opened from main ImageJ toolbar when required.
Appendix iv. Publications

