PATHOLOGICAL ROLE OF DOUBLE-STRANDED DNA ANTIBODIES IN MULTIPLE SCLEROSIS

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ABSTRACT

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Multiple sclerosis is a complex disease and one for which the aetiology remains largely unanswered. Anti-dsDNA antibodies have been found intrathecally and bordering lesions in multiple sclerosis patients and in view of their known pathogenity in lupus nephritis the aim of this project was to further investigate their role in multiple sclerosis. Using the acute experimental allergic encephalomyelitis (EAE) model in the Lewis rat, the inflammatory phase of disease was profiled using immunohistological and ELISA methods and was related to clinical sign severity. The parameters of interest were central nervous system deposits of IgM, IgG, B cells and C3 and anti-DNA antibodies in sera, cerebrospinal fluid and *in situ*. *In situ* evaluation of anti-dsDNA antibodies was also performed in tissue taken from Biozzi (AH) mice (relapsing/remitting EAE model) and from a multiple sclerosis patient. Inflammatory deposits specifically at sites of perivascular cuffing were found to increase with increasing clinical sign severity. At the time clinical signs had plateaued in the Lewis rat, intrathecal anti-dsDNA antibodies were at their highest level and anti-ssDNA antibodies at their lowest. The latter possibly due to their involvement in the 'clearing-up' process following tissue damage. Using novel DNA probes fluorescence suggestive of the presence of anti-dsDNA

antibodies was seen in both animal and human tissue. Within human tissue the antibodies appeared to accumulate around active lesions and within vessels, raising the question of these antibodies having differing location dependent functions. EAE models have the potential to investigate these findings further and to evaluate new therapies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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ABBREVIATIONS

ABH	Antibody high
ADP	Adenosine diphosphate
APES	Aminopropyltriethoxysilane
APOE	Apolipoprotein E
BSA	Bovine serum albumin
cDNA	Complementary DNA
CJD	Creutztfeldt Jakob disease
CNS	Central nervous system
CNP-1	2′,3′-cyclic nucleotide 3′-phosphodiesterase
CRYAB	Crystallin, alpha B
CSF	Cerebrospinal fluid
CV	Coefficient of variant
DAB	3,3-diaminobenzidine
DAPI	4’,6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
EAE	Experimental allergic encephalomyelitis
EB	Elution buffer
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FA	Freund’s adjuvant
FCA	Freund’s complete adjuvant
FIA	Freund’s incomplete adjuvant
GFAP	Glial fibrillary acidic protein
H&E	Haematoxylin and eosin
HCl	Hydrochloric acid
HES5	Hairy and enhancer of split 5 (Drosophila),
HRP	Horseradish peroxidase
HLA	Human leukocyte antigen
HPLC	High performance liquid chromatography
IEF	Isoelectric focusing
IFN	Interferon
Igs	Immunoglobulins
IL	Interleukin
IMS	Industrial methylated spirits
IVIg	Intravenous immunoglobulins
LFB	Luxol fast blue
LPS	Lipopolysaccharide
LY1	Lymphocyte antigen 1
MAG	Myelin-associated glycoprotein
MHC	Major histocompatibility complex
mIg	Membrane immunoglobulin
mIgM	Membrane IgM
MMR	Measles mumps and rubella
MOG	Myelin oligodendrocyte glycoprotein
MPB	Myelin basic protein

MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
NAA	N-acetylaspartate
NO	Nitric oxide
OD	Optical density
OPD	o-phenylenediamine
PBS	Phosphate buffered saline
PE buffer	Protein extraction buffer
PLP	Proteolipid protein
PTPRC	Protein-tyrosine phosphatase receptor type C
PVC	Perivascular cuffing
ROHP	Reverse osmosis high purity
rpm	Revolutions per minute
se	Standard error
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
ssDNA	Single-stranded DNA
Syk	Spleen tyrosine kinase
TdT	Terminal deoxynucleotidyl transferase
TE buffer	Tris-EDTA buffer
TGFβ	Transforming growth factor
TNFα	Tumour necrosis factor alpha
TNFβ	Tumour necrosis factor-beta
UVB	Ultraviolet B
vCJD	Variant Creutztfeldt Jakob disease

1 CHAPTER 1: INTRODUCTION

Multiple sclerosis is a debilitating disease that affects young adults usually before the age of 40, with women being at greater risk than men [Homo-Delarche *et al*. (1991)]. The progressive neurological impairment, which frequently develops during the course of the disease, is likely to be the result of irreversible tissue injury [Trapp *et al*. (1998)]. Although the disease pathology is thought to be due to an uncontrolled immune system creating an autoimmune state, leading to organ specific autoimmune destruction of CNS myelin [Sørensen & Ransohoff. (1998)], the aetiology remains largely unanswered.

MS symptoms are far ranging and include visual disturbances, abnormal eye movements, facial weakness, impaired hearing and balance, cognitive disturbances, psychological problems such as depression, unpleasant sensations, paralysis/numbness and autonomic effects such as incontinence [www.patient.co.uk]. The severity and frequency of MS symptoms vary from patient to patient and have a variable and often unpredictable progression.

The complexity of the disease and the possibility that MS itself may be a number of different diseases which are lumped together under the one umbrella, has meant that it remains largely incurable, with treatments concentrating on minimising pain and decreasing the duration and frequency of episodes [www.patient.co.uk]. Research using various

animal models has suggested that there are different pathological end-points to MS and that the course of the disease may depend on the initial trigger and the individual's genetic make-up [Prat & Martin (2002)]. The two most common types of MS are relapsing-remitting and chronic progressive.

The relapsing-remitting type of MS is the most common type, affecting approximately 80% of patients and is the type that most people consider to be MS [Evans & Schriver (2002)]. This form of the disease is characterised by relatively mild, short-lived symptoms (flares) which disappear on their own accord, leaving the sufferer symptom free for varying lengths of time, weeks, months, or in some cases years [Evans & Schriver (2002)]. This type of the disease tends to affect people before the age of 40. In contrast, the chronic-progressive type of MS tends to affect people over the age of 45. This type of MS can be further split into two sub-types; primary-progressive and secondary-progressive [Evans & Schriver (2002)]. Primary-progressive MS affects about 10% of people with the disease and is characterised by the progression of symptoms without periods of remission. Secondary-progressive MS affects about 50% of people initially diagnosed with the relapsing-remitting form of the disease. This sub-type of MS very rarely involves periods of remission [www.mult-sclerosis.org].

Symptoms expressed by MS patients are largely due to impaired neural transmission. In MS the myelin sheath, which surrounds the nerve fibre

becomes damaged, this causes the nerve impulses to slow down to about 1 m/s compared to up to 120 m/s for a myelinated fibre; speed being dependent on the total thickness of the fibre [Hess (1997)]. In severely demyelinated nerve fibres conduction is completely prevented. This effect is resolved relatively quickly, after a few days, by the formation of sodium channels in demyelinated internodia [Hess (1997)]; this eventually results in the remission of clinical signs of the disease. In addition to demyelination of the nerve fibres MS is characterised by the presence of plaques (lesions) in the CNS, of which there are four types. Shadow plaques are plaques that have reduced myelin density resulting in thin myelin sheaths, which are undergoing remyelination. They have dense astroglial scar formation and are typically found in the brains of patients with acute or chronic progressive MS [Kesselring (1997)]. Concentric plaques are rare and tend to be located around a central blood vessel. They are made up of alternating rings of myelinated and demyelinated tissue and are found in MS patients suffering from acute and subacute MS. Destructive plaques are found in severe cases of acute and subacute MS. These plaques may be characterised by loose tissue, dilated extracellular space and can lead to the degeneration of the CNS. Plaques involving the destruction of astrocytes are rare and are only found in Caucasian MS patients with acute and progressive MS. These plaques are found in the spinal cord and are devoid of astrocytes, myelin and oligodendrocytes. Axon fibres are present and can be remyelinated [Lassmann (1997)].

1.1 Aetiology

MS is a T cell mediated autoimmune disease [Khoury *et al*. (1996)] for which the disease process is thought to comprise of an induction phase, followed by an effector phase involving the migration of activated T cells into the CNS. The disease is characterised by perivascular T cells, particularly myelin epitope specific $CD4^+$ T cells [Ota *et al*. (1990); Bernard & Kerlero de Rosbo (1991); Mendel *et al*. (1995); Kerlero de Rosbo *et al*. (1995); Zamvil & Steinman (1990)], B cells and macrophages infiltrating the blood brain barrier leading to demyelination within the CNS, resulting in impaired nerve conduction and paralysis [Alvord (1984); MacFarlin & MacFarland (1983); Yednock *et al*. (1992); Karin *et al*. (1993)]. It is thought that the overall phenotype of the immune response determines the expression pattern of clinical and pathological disease [Chitnis *et al*. (2001)].

Demyelination of the brain and spinal cord is characterised by plaques and an associated inflammatory response consisting of perivascular infiltrates of B cells, T cells and macrophages within the lesion and immediately surrounding its borders [McDonald & Ron (1999); Trapp *et al*. (1998)]. Plaques result from the attempts made by oligodendrocytes and astrocytes to repair damaged myelin, which results in the production of scar tissue, known as gliotic plaques. These plaques harden and become sclerotic and interfere with or obstruct the flow of nerve impulses that pass along the nerve cells. Remyelination is partially possible if inflammation is quickly managed, for example by the administration of steroids. If the

inflammatory process is left unmanaged, then the beneficial effect of remyelination is prevented by the gliotic plaque forming a barrier between myelin producing cells and axons. The position of the plaque within the CNS determines the symptom, if several plaques are active at the same time they will produce multiple disturbances.

Analysis of cerebrospinal fluid samples has indicated that MS is typified by intrathecal IgG synthesis, with high affinity anti-DNA antibodies being a major component of this response [Owens *et al*. (1998)]. B cell populations in CSF and brain plaques have been found to be of restricted diversity suggesting that those mediating the response in MS are seen to be compartmentalised within the CNS and are not well represented in peripheral circulation [Owens *et al*. (1998); Colombo *et al*. (2000); Qin *et al*. (1998); Baranzini *et al*. (1999)]. Active demyelinating MS plaques have an increased B cell content [Esiri (1977)] with IgG deposition, including anti-dsDNA antibodies around the borders which correlate with the presence of activated complement fragments and complexes, compared to that found in older inactive lesions [Woyciechowska & Brzosko (1977); Lumsden (1971); Storch *et al*. (1998)]. This association of antibodies and active lesions has led to the hypothesis that the surrounding antibodies may be responsible for plaque formation [Archelos *et al*. (2000)]. It is possible that anti-dsDNA antibodies may be involved in increasing the size of the plaque from the border region by increasing the rate of localised apoptosis. Studies *in vitro* have shown that anti-dsDNA antibodies from patients with SLE significantly increase neutrophilia apoptosis, compared

to the rate of neutrophil apoptosis in controls [Armstrong *et al*. (2006)]. If anti-dsDNA antibodies can have this effect in SLE it is possible that they may influence the apoptosis rate of other cell types and this may happen in MS. Alternatively, apoptosis could be responsible for the induction of anti-dsDNA antibodies. It has been hypothesised that in diseases, such as SLE, the clearance of apoptotic cells may be much slower than that in healthy people and this may result in lingering nucleoproteins inducing an autoimmune response [Herrmann *et al*. (1996)]. The importance of anti-dsDNA antibodies in the MS autoimmune repertoire has not yet been fully investigated and they are not considered to be a reliable clinical biomarker. This is in contrast to SLE for which these antibodies have been found to be a relatively robust predictor of the disease [Kumar *et al*. (2009)]. In the past MS research has focused on other autoantibodies thought to be linked to MS such as myelin oligodendrocyte glycoprotein and 2′,3′-cyclic nucleotide 3′-phosphodiesterase [Williamson *et al.* (2001)].

1.1.1 Familial Genetics

Considerable research has been performed with regard to MS. Such research has used a number of animal models and various induction agents; myelin peptides [Asensio *et al*. (1999)] and viruses [Miller *et al*. (1997)] being frequently employed to induce the disease-state. The results from such studies have been convincing in many cases, conflicting in others, but overall demonstrate the complexity of the disease, or possibly diseases and the elusiveness of the pathogenic agent. The following discusses genetic links that may make some people more susceptible to MS than others.

Prevalence of MS globally was estimated to be 0.1% in 2006 [www.mssociety.org.uk]. Although MS is not strictly an inherited disease, there does appear to be a genetic link, with children of one parent with MS having a 2% chance of developing the disease, compared to 0.1% for the general population who have no known relatives with the disease [www.mssociety.org.uk]. This risk is further reduced to levels comparable with those seen in the general population if the child is male, as females are twice as likely to contract MS [www.mssociety.org.uk]. The risk of developing MS appears to be the same regardless of whether the mother or father is affected [Multiple Sclerosis Genetics group (1998)] and is more frequent among direct relatives of probands, becoming less frequent as the relationship to the proband becomes more remote [MacKay & Myrianthopoulos (1966)]. If both parents have MS, then the chance of their offspring developing MS increases to 20-30%. Studies have found concordance to be higher for sister pairs than for brother pairs [Multiple Sclerosis Genetics group (1998)] and comparisons of MS development between step-brothers and sisters (half siblings) compared to siblings from the same mother and father (full siblings) show that full siblings are nearly three times as likely to show concordance for MS that that for half siblings [Sadovnick *et al*. (1996)].

Twin studies have been extensively used to further support the familial link to MS, but are not without limitations. Twins are difficult to study in any large number and some of the studies which have been performed suggest that concordance of MS in twins is extremely rare and as such

point to another trigger factor independent of the genetic make-up, e.g. infection in childhood [James (1996); Bobowick *et al*. (1978)]. In contrast, other researchers have found there to be a strong case for a genetic link by examining twins, in particular monozygotic twins for which a 25% concordance rate has been found compared to 3.79% for dizygotic twins [Hawkes (1997)]. A Canadian study [Ebers *et al*. (1986)] which looked at the concordance of MS in monozygotic and dizygotic twins also found concordance of 26 and 2%, respectively.

Population genetics has found that there are some communities that are more susceptive to developing MS than others. One such community can be found in the south-western part of the Sassari province of Sardinia. This particular area comprises the Common Logudorese Linguistic domain, which is known to be genetically stable population with high inbreeding rates compared to other areas. In comparison, the neighbouring Catalan area, which is linguistically and genetically distant from other Sardinian domains, MS susceptibility is no higher than would be expected for this latitude [Pugliatti *et al*. (2002)].

Despite a considerable amount of research focusing on finding a specific genetic link for MS, no one underlying risk gene has been identified and proven to be fully responsible for MS pathology. However, results from familial studies do undoubtedly show that a genetic link exists and that this link becomes stronger the closer the relationship to the proband. What is also clear is that MS shows some characteristics that are consistent with a

polygenic model. A polygenic model of inheritance is one for which each contributing gene has small and relatively equal effects, the effects of each allele are additive and there is no dominance. MS however contradicts this model in that it is thought to depend upon epistasic effects, i.e. the modification of one gene by others and this certainly appears to be the case with regards to the regulation and severity of the disease [Vyse & Todd (1996)]. Secondly, MS is not entirely dependent on genetics. This is supported by most monozygotic twins being discordant for MS, which implies that non-genetic risk factors e.g. environmental factors play a significant role in MS.

So, although a familial genetic link exists, this link on its own is not strong enough to be considered reliable for use in clinical diagnosis of MS or in the prediction of developing the disease.

1.1.2 The human leukocyte antigen

MS genetics focuses on the major histocompatibility complex whose molecules present antigens to T cells. The MHC in humans is known as the human leukocyte antigen system. In MS, susceptibility is linked to class II MHC genes with the HLA type often being associated with ethnicity. For Caucasians the strongest genetic link in MS is the HLA-DR2 haplotype; individuals having this haplotype are four times more likely to develop the disease compared to HLA-DR2 negative people [Fierz (1997)]. Familial studies [Francis *et al*. (1987)] have confirmed there to be a linkage with the HLA-DR2 allele and suggest that this linkage is responsible for the genetic aetiology in 16 - 62% of MS cases. However,

the absence of this link in other MS cases supports the heterogeneous nature of this disease and the possibility of external triggers or the presence of a predisposing haplotype.

Links to the MHC have been found in studies of Canadian and Finnish MS populations [Lincoln *et al*. (2005)]. Strong associations were found for blocks in the HLA class II region, these being the cell surface glycopeptide antigens of the HLADP, DQ and DR loci. The strongest associations were however found for HLA-DRB1. The results show that the MHC-associated susceptibility of MS is determined by HLA class II alleles, allelic interactions and closely neighbouring variants. This is supported by Chao *et al*. (2008) who concluded that HLA-DRB1*15 allele is only partly responsible for MS susceptibility and in addition requires either epistatic interactions, epigenetic modifications of some haplotypes or neighbouring structural variation.

It has been reported that the frequency of specific T cells to the amino acids 95-116 in the proteolipid protein (PLP95-116) is significantly elevated in MS patients who carry class II major histocompatibility type HLA-DR2^{+}, compared with those who are HLA-DR2^{-} [Ohashi *et al*. (1995)]. However, it remains unclear whether HLA-DR2 restricted T cells alone can induce MS, or if other cell types such as B cells are necessary [Kawamura *et al*. (2000)].

Studies [Zipp *et al*. (1995)] have found that T cells from people positive for HLA-DR2 produce more TNFβ and TNFα than those taken from people negative for HLA-DR2 which increases their risk of inflammatory disorders. The reason for this may be due to the presence of a polymorphic gene in this region. A TNFα-376 promoter polymorphism association has been found in Spanish populations of MS which appears to be independent of MHC class II. This risk is synergistically increased in the presence of HLA-DRB1*1501 which appears to be linked to TNFα polymorphism [Fernandez-Arquero *et al*. (1999)]. This finding however was not found to be the case in Northern European populations [Weinshenker *et al*. (2001)] and was further dismissed as being the sole MHC determinant in northern European populations by Ligers *et al. (2001).*

Further studies [Francis *et al.* (1987)] support the association of a MS susceptibility gene residing within the HLA-D region and others [Barcellos *et al.* (2003)] have found HLA-DR2 to have a dose-response effect on MS susceptibility, with two copies of this haplotype increasing the risk of disease susceptibility and severity.

The HLA-DR13 haplotype has been found not only to be associated with an increased susceptibility to MS, but also with disease progression. It was found that in a population of Italians residing in the north-east of the country that the HLA-DR13 haplotype was found in 40% of patients with relapsing-remitting MS and in just 4% of those with secondary progressive MS. The haplotype was also found in 16% of the population studied who

did not exhibit MS [Perini *et al*. (2001)]. These findings suggest that HLA-DR13 may have a protective effect against the more severe form of the disease or is associated with a milder version.

Evidence that the MHC class I HLA-C gene influences susceptibility to MS was found by the International Multiple Sclerosis Genetics Consortium (2007). The results showed there not only to be a significant link with the HLA-C locus, but also that the allele HLA-C*05 potentially had a protective effect against MS due it being under represented in the MS patients on the study when compared to the control group.

In addition to HLA-DR2, studies have found MS associations with other HLA types such as HLA-A3, HLA-B7 and HLA-Dw2, the latter showing a strong association with MS [Terasaki *et al*. (1976)]. Support for the HLA-Dw2 allele has come from epidemiology studies [Kutzke and Hyllested (1979); Shermata *et al*. (1985)] which have found that in general, MS is linked to latitude and the exceptions to this e.g. Japan, can be explained by a lack of the Dw2 allele.

Studies [DeLuca *et al*. (2007)] have suggested that the DRB1*01 allele has a protective effect against MS. It was also shown to modify disease progression as seen in Sardinian MS patients in which nineteen patients exhibiting relapsing-remitting MS had the DRB1*01 allele, in contrast to patients with the progressive form of MS for whom this allele was absent.

From the vast number of investigations into finding a gene responsible for MS susceptibility within the MHC two main points can be concluded. The first being that there does appear to be a genetic association with MS susceptibility which resides within the HLA and secondly it is apparent that multiple alleles are involved, some of which e.g. HLA-C*05, can be seen to have a protective effect against the disease.

1.1.3 Other genes

With the advancement of technology it has become apparent that the genetic risk of MS is due to numerous genetic variants. More recently (2005), a high power admixture mapping scan has found a locus on chromosome 1 that is significantly associated with multiple sclerosis [Reich *et al*. (2005)]. Admixture mapping is method where by the genome can be scanned for gene variants that are potentially pathogenic in complex diseases such as MS. A genomewide association study [The International Multiple Sclerosis Genetics Consortium (2007)] found that single-nucleotide polymorphisms in genes encoding for IL2Rα and IL7Rα chains are associated with MS. IL2Rα has also been associated with type 1 diabetes and Graves disease and goes some way in supporting MS as being an autoimmune inflammatory disease, with the presence of IL2Rα being an indication of susceptibility.

IL7R is another gene, which has been found to have an association with MS susceptibility. The association between the SNPs in this gene and multiple sclerosis has been supported by a number of studies. One study

[Gregory *et al*. (2007)] found that the SNP rs6897932 had a significant association with MS in European and Nordic populations. Whereas another study [O'Doherty *et al.* (2008)] found that such an association was dependent on geographical location, with a positive association being found for patients from Olmsted County, Minnesota, but not for those in Belfast, Northern Ireland.

T cell receptor genes have been shown to increase susceptibility to MS. Studies [Utz *et al*. (1993)] in which monozygotic twins concordant or discordant in response to self and foreign antigens were exposed to myelin basic protein or tetanus toxoid. Researchers found that the controls and concordant twins selected similar V-alpha chains, whereas those discordant for MS selected a different set of T cell receptors. It is thought that the T cell repertoire of a person dictates their susceptibility to disease and that this in turn may be affected by environmental factors.

Studies have found that a diverse T cell repertoire increases the risk of MOG-induced EAE relapse. Terminal deoxynucleotidyl transferase (TdT) is a specialised DNA polymerase which is expressed in immature pre-B and T cells; it aids T cell diversity by catalysing the random insertion of non-template nucleotides. A lack of TdT leads to low T cell diversity. Using $TdT^{-/-}$ (low diversity) and $TdT^{+/+}$ (high diversity) mice it was found that $TdT^{-/-}$ mice immunised with MOG did not relapse following a single episode of EAE, $TdT^{+/+}$ mice on the other hand displayed successive EAE episodes. In this study there was no evidence of epitope spreading and

the relapse was suggested to be due to the highly diversified T cell repertoire of $TdT^{+/+}$ mice which resulted from the initial MOG-specific T cells. It was proposed that during the first episode of EAE the T cell response is down regulated in some individuals. However, in individuals with a highly diverse T cell repertoire, as seen in $TdT^{+/+}$ mice another subset of specific T cells expands which leads to relapse in the absence of epitope spreading [Fazilleau *et al*. (2007)].

It has been proposed that the MBP gene is genetically linked to and/or associated with MS. A study [Tienari *et al*. (1992)], using a population in Finland for which there was a hotspot of familial clustering, found that the allele frequencies significantly differed between individuals with MS and the negative controls. In particular this difference related to a 1.27-kb allele, which was seen to occur at a higher incidence in MS patients. A genetic linkage was found between MS susceptibility, MBP and HLA. Other studies [Rose *et al*. (1993); Eoli *et al*. (1994)] found there to be no such linkage or association. It was hypothesised by Tienari *et al.* (1992) that the role of the MBP gene in MS may be due to a functional defect, possibly producing low levels of MBP expression and thus impairing the ability of the nerves to remyelinate.

Studies have also found there to be associations with an increased frequency (28%) of the K469 allele of the intercellular adhesion molecule-1 [Mycko *et al*. (1998)] and the presence of the interferon-gamma gene on chromosome 12q14 [Vandenbroeck *et al*. (1998)].

Other genetic studies have suggested that a point mutation in the gene encoding protein-tyrosine phosphatase, receptor-type C (PTPRC also known as CD45) may be involved in disease development. The mutation lies within exon 4 of the gene and interferes with mRNA splicing, resulting in altered expression of CD45 isoforms on immune cells. Jacobsen *et al.* (2000) found that, in three out of four independent case-controlled studies, the frequency of this point mutation was higher in MS patients than in people without MS. This mutated gene is thought to alter the expression of CD45 proteins on lymphocytes and may increase susceptibility to the disease possibly by affecting activation, adhesion or migration of immune cells. These findings are far from conclusive, with the results being positive for German based studies, but not so for studies using patients from the United States. Jacobsen suggests that environmental factors may partially explain this finding.

The genes mentioned in this section are just a handful that have shown some association or linkage with MS, many more have been implicated in this way. It is clear that genetics does have a role to play in MS susceptibility and disease progression and that multiple genes are involved in this process. However, with continual contradictory findings it is unlikely that any one set of genes is exclusively responsible for the development of MS.

1.1.4 Genetic modifiers

Not only are genetics involved in an individual's susceptibility to MS, they are also thought to influence the severity of the disease. Studies have

used MRI technology to investigate disease severity between familial MS and non-familial MS. They found that familial MS individuals had significantly more destructive lesions, lower volume of whole brain, white matter and grey matter and other indications of greater brain degradation. This study found that the closer the family relationship the greater the damage to the grey matter, suggesting that there is not only an increase in MS susceptibility risk associated with genetics, but also a link with severity [Zivadinov (2007)].

A genetic link with disease severity is supported by a study in which early death in MS was found to be linked with the CCR5delta 32 deletion allele. It was found that MS patients with this deletion were twice as likely to die compared to individuals without it. In addition, in female patients this risk was more pronounced [Gade-Andavolu *et al*. (2004)].

The apolipoprotein E (APOE) gene has been implicated in the severity of MS by a number of researchers. Studies have found an association between the APOE4 allele and an increase in disease severity and faster progression. One study [Chapman *et al*. (2001)] found that the presence of the APOE4 allele was associated with a faster progression of MS but had little involvement with regards to age of onset. It was hypothesised that APOE4 affects the efficiency of neuronal maintenance and repair mechanisms, which in turn results in the disease state. Studies [Schmidt *et al*. (2002)] conducted to further investigate the role of APOE looked at its functional alleles and seven SNPs which are within 13 kb of the APOE

gene. Using familial association analysis, one of the seven SNPs was found to increase MS susceptibility and that people carrying the APOE4 allele were prone to a greater severity of MS. It was also found that women with the APOE2 allele developed MS of less severity. Another study [Enzinger *et al*. (2003)] found that the APOE4 allele was linked to a decrease in N-acetylaspartate (NAA):creatine ratio compared to controls without this allele. NAA is found in mature neurones, a decrease in this is associated with neuronal and axonal loss resulting in neuronal dysfunction. It was found that the lower the NAA:creatine ratio the higher number of relapses and the faster the disease progressed.

Studies [Schrijver *et al*. (1999)] have found there to be a higher rate of disease progression in patients who have the IL1TN*2 allele and lack the IL1B*2 allele, compared to those not having this allele combination.

1.1.5 Epidemiology

Although genetic make-up may influence an individuals susceptibility to MS and disease progression, it is widely accepted that genetic make-up alone is not sufficient to induce MS and that an external trigger is also required.

Environmental factors, in particular geographical location have been recognised to increase a person's susceptibility to MS. Prevalence of MS at the equator is extremely low, whereas the further away from the equator, in particular at latitudes higher than 40°, an increased rate of the disease is observed. Twin studies [Islam & Mack (2007)] suggest that

exposure to sunlight in childhood has a protective effect against MS. It is possible that vitamin D synthesised in response to ultraviolet radiation from the sun has the ability to inhibit an inappropriate immune attack. Other possibilities as suggested in migration patterns and epidemiological studies have shown that people who are born in an area of the world with a high risk of MS and then move to a low risk area acquire the risk of their new home if the move occurs before the age of 15 [NMSS Information Resource Center and Library (1997)]. This supports the theory that the trigger factor in MS occurs early on in life, with a viral component e.g. measles being a likely candidate. Alternatively it could be the way the T cell repertoire is developed.

The measles virus has been identified as a potential MS trigger due to high measles virus antibody titres found in MS patients. In addition there is an increased incidence of MS in people who have contracted measles late in life compared to those who contracted the virus in early childhood. This is supported by the observation that geographical areas where MS is rare also have a high incidence of childhood measles, i.e. the late onset of measles is low. It is possible that susceptibility to MS is due to the difference in the person's immune response to measles due to their age at the time of infection [Alter (1977)]. The vaccine against measles became available in 1968, however it was not until the introduction of the MMR vaccination in 1988 that routine immunisation took place in the UK. As the onset of MS is typically between 20 – 40 years of age it is still too early to assess the potential beneficial impact of vaccination on this disease.

One disease, although rare in occurrence (1 in 8000 for the people who contract measles before they are 2 years of age), for which a decline in incidence has been observed since the introduction of the measles vaccine is subacute sclerosing panencephalitis. This disease causes brain degeneration and ultimately death. It is linked to exposure to the natural measles virus early on in life (under 2 years of age), with symptoms typically appearing before the age of 20. The measles vaccine appears to prevent this in some way, which is not fully understood [Miller *et al.* (1992)].

If early contact with the measles virus has a protective effect against MS, then we can expect to see a gradual decrease in the prevalence of the disease in developed countries due to the introduction of the measles vaccine. At present there appears to be little sign of this, in fact over the past 10 years or so MS has increased this however may be due in part to improved diagnostic skills and improved life expectancy for those with the disease.

Diet is another factor that may be linked to MS susceptibility. One theory for this is based on the observation that areas in the world with increased MS have an incidence that is closely related to the consumption of saturated fats. Studies have shown that levels of essential fatty acids in the blood tend to be low in some people with long standing MS [Marshall (1991)] Studies which have investigated the effect of lipids on the immune system and autoimmune disease have found that in general, n-3 fatty

acids and essential fatty acid deficient diets worsen T-cell mediated diseases such as MS and n-6 fatty acids have the ability to prevent or decrease the severity of such diseases. It is thought that the modifying effect of essential fatty acids on immunoregulation and inflammation is due to their effect on cytokines through the production of eicosanoids, although they also have a role with the regulation of gene expression and signal transduction pathways [Harbige (1998)]. Support for the preventative effects of a diet high in essential fatty acids is found in communities living along the coastlines of counties such as Japan and Norway. In these areas the typical diet is fish and as such is high in essential fatty acids, here the incidence of MS has been found to be much lower than would be expected in the general population at these latitudes [Challem (1994)].

Other dietary components that have been linked to the development/disease progression of MS are B12 deficiency, excess sugar intake and folic acid deficiency [Challem (1994)]. Recently (2008) it has been proposed that vitamin B12 deficiency may exacerbate symptoms of MS. It has been reported that a patient suffering from MS whose symptoms had rendered her wheelchair bound was treated for vitamin B12 deficiency following which there was a 60% improvement in symptoms within 2 days of treatment [Kayalackakom (2009)]. This finding is unlikely to be of benefit to the majority of MS sufferers, but for some, in particular those with chronic MS symptoms it is possible that effects due to vitamin B12 deficiency may be overlooked. Treatments with vitamin B12 supplements may appear to have the effect of alleviating the MS

symptoms, but this may be alleviating the vitamin deficiency symptoms sometimes associated with MS, rather than the actual MS symptoms.

There is no doubt that external factors play an important role in MS. Geographical location pre-disposes an individual to MS, whether this is due to the presence of a viral/bacterial component in that region or local dietary habits. What is also clear is that the timing of exposure to environmental factors is important, whether it is the proposed protective effect of UV light in early childhood or the timing of exposure to a childhood disease.

1.2 Pathological triggers

The most likely trigger for MS and one for which epidemiological studies have provided strong evidence, is that of a viral or bacterial infection [Kurtzke (1993)]. No specific virus or bacterium has been conclusively identified in this role, but a number of mechanisms of how such an encounter may result in autoimmunity have been postulated, these being molecular mimicry and epitope spreading.

1.2.1 Epitope spreading

Epitope spreading is the development of an immune response to epitopes, which are distinct from and non-cross-reactive with the disease causing epitope. Epitope spreading may be more relevant in diseases that are caused by chronic infection of the target organ and in the chronic stage of an autoimmune process. In MS, antibodies may initially target myelin basic protein, however with the destruction of the myelin sheath other

antigens are released e.g. myelin oligodendrocyte glycoprotein, resulting in additional antibody types becoming involved in the disease process (spreading), which may be far removed from the initial trigger. This is known as intermolecular epitope spread. Intramolecular epitope spread occurs when antibodies are directed against a particular amino acid sequence of an antigen e.g. an initial response against MPB amino acid sequence 82-99 spreads to a different epitope such as MBP 102-118. The initial antibody response results in the destruction of myelin, exposing 'hidden' antigenic sequences, which then come under attack and lead to an acceleration of organ specific destruction [Coyle (2003)].

Epitope spreading has been demonstrated in a number of animal models, in particular murine EAE models. Such research has supported the role of epitope spreading in autoimmune disease and also that epitope spreading can be T cell mediated. Research has found that with regards to the EAE model, myelin damage is necessary for the initiation of epitope spreading and also that epitope spreading is required for relapse to occur in the EAE model [Powell & Black (2001)].

In an EAE model using PL/J mice immunised with MBP, it was hypothesised that reactivation of EAE by the superantigen staphylococcal enterotoxin causes the intramolecular spread of T cell specificities for other MPB epitopes [Torres *et al*. (2001)].

1.2.2 Molecular mimicry

Molecular mimicry is the activation of autoreactive T cells following an encounter of a pathogen with epitopes that are shared or cross-reactive with self-antigens [Fujinami & Oldstone (1985); Oldstone (1987)]. It is possible that molecular mimicry could trigger the initial activation of autoreactive T cells and/or induce the expansion of a memory T cell population, while superantigens could reactivate autoreactive T cells and induce relapses. Superantigens are immunostimulatory bacterial or viral toxins that bind to MHC class II molecules and T cell receptors. They are active at very low concentrations and are able to interact with MHC class II molecules outside the binding groove without compromising their stimulatory effect on T cells. There are three main classes of superantigens. The first are endogenous superantigens, which are encoded by viruses and are integrated into the genome e.g. Epstein-Barr virus. The second are exogenous superantigens, which are secreted by bacteria e.g. staphylococcal pyrogenic exotoxins. The third class are the B cell superantigens, which mainly stimulate B cells e.g. staphyloccal protein A [Solanki *et al*. (2008)]. Superantigens have a role in molecular mimicry and MS when their antigens are similar to that of self-antigens.

Molecular mimicry has been demonstrated in a number of murine models, in which mice, expressing virus proteins as transgenes, develop autoimmune disease following viral infection [Ohashi *et al*. (1991); Oldstone *et al*. (1991)]. Theiler's murine encephalomyelitis virus [Miller *et al*. (1997)] is a naturally occurring mouse pathogen that induces chronic

$CD4^+$ T cell mediated demyelinating disease. This virus remains within the CNS causing virus specific $CD4^+$ T cells to initiate the demyelination process. This EAE model not only provides an example of how a chronic CNS infection can result in the priming of self-reactive antigens that initiate demyelination, but also demonstrates that myelin specific T cells are not essential in the pathogenesis of the disease [Miller *et al*. (1997)]. Such studies show that immunisation using CNS material is not exclusive in producing EAE signs and that infection by some bacteria or viruses can also initiate the disease process. This supports the hypothesis that myelin antibodies are produced in response to demyelination initiated by an alternative mechanism or by antibodies other than those targeted towards myelin peptides, this may also be true in the case of anti-dsDNA antibody induction.

The presence of anti-dsDNA antibodies in MS patients may also be explained using the molecular mimicry hypothesis. It would be reasonable to assume that anti-DNA antibodies are produced following inflammation, during which cells are destroyed releasing large amounts of DNA. However, studies have shown that mammalian DNA, even in the presence of an adjuvant has an extremely low antigenic potential [Gilkeson *et al*. (1991)]. Based on this assumption it is quite possible that anti-dsDNA antibodies may be the result of bacterial infection.

It has been demonstrated that bacterial proteins and bacterial polysaccharides can induce anti-dsDNA antibodies in a number of

experimental models [Madaio *et al*. (1984); Gilkeson *et al*. (1989); Desai *et al*. (1993); Rekvig *et al*. (1995); Ray *et al*. (1996)] and that these peptides can be molecular mimics of dsDNA. A study whereby pre-autoimmune NZB/W mice (mice which spontaneously produce anti-DNA antibodies as they age) immunised with *Escherichia coli* dsDNA subsequently produced antibodies resembling spontaneous autoantibodies that bind to mammalian dsDNA [Wloch *et al*. (1997)]. The same study when performed using normal mice found that the induced antibodies only bound to bacterial dsDNA, suggesting that a genetic component is important in disease initiation. In studies investigating SLE using MRL/MpJ mice, it has been found that repeated administration of bacterial plasmids containing eukaryotic promoter elements may induce immune responses with the generation of antibodies cross-reacting not only with the mammalian DNA, but also more significantly with nuclear antigens [Radic *et al*. 1993]]. Gilkeson *et al.* (1989, 1991) proposed that bacterial DNA could be the immunogen that induces the production of antibodies that are reactive to DNA in patients with SLE, this also being a possibility in MS.

Although studies have found a restricted antibody presence in the CSF of MS patients, to-date none of these antibodies have been shown to be solely responsible for the disease state. Myelin antibodies have been well documented and studies [Tuohy *et al*. (1997)] investigating these antibodies have shown that epitope spreading occurs during MS, thus changing the target slightly during the course of the disease. However,

although anti-myelin antibodies are found in MS patients and are considered by many to play an important role in the pathology of MS, a number of these antibodies have also been found in normal individuals and in diseases where demyelination is not seen [Rauer *et al*. (2006)]. In the Lewis rat model of EAE, although a single injection of myelin or complete spinal cord, in the presence of an adjuvant, produces marked clinical signs of EAE and a myelin antibody response, demyelination is not observed [Linington *et al.* (1993)]. This would suggest that although myelin antibodies may have a place in MS, they are unlikely to be entirely responsible for the pathogenesis of the disease.

Controversially, it has been proposed that variant Creutztfeldt Jakob disease (vCJD) is an extreme progressive form of MS [Hughes *et al*. (2001); Ebringer *et al*. (2005); Wilson *et al*. (2004)]. The symptoms (visual disturbances, impaired motor co-ordination) certainly have many similarities with MS and in the early stages of vCJD, sufferers are often mis-diagnosed as having MS. Interestingly, studies have found that antibodies to a common soil bacterium (*Acinetobacter*) and to a lesser extent to the microbe *Pseudomonas*, are frequently found in significantly higher titres in people with MS than in people without the disease. The same has been found to be true of cows with bovine spongiform encephalopy. It is hypothesised that molecular mimicry is responsible, as *Acinetobacter* have epitopes similar to those found in nervous tissue and there are similarities in the amino acid sequence of the bovine prion sequence (RPVDQ) and an *Acinetobacter* molecule. It is suggested that

antibodies formed in response to the *Acinetobacter* antigen could lead to the activation of auto-reactive cells and hence the production of autoantibodies, in particular those which target the CNS, resulting in disease development [Hughes *et al*. (2001); Ebringer *et al*. (2005); Wilson *et al*. (2004)]. In support of this theory it has been found that 50% of MS patients suffer from sinusitis which could be the result of *acinetobacter* infection [Gay *et al*. (1986)]. Such research provides a convincing case for the role of molecular mimicry between *Acinetobacter* and brain antigens and this may also have a role in CJD and MS. Although this is an interesting theory and one that provides a possible pathogenic link between MS and CJD, the prion hypothesis remains the most widely accepted pathogenic factor in the overall pathology of CJD.

1.2.3 Hormones

Hormones have been implicated in the control of MS due to the disease predominating in young women. During pregnancy, clinical signs of MS are seen to subside, this is in contrast to postpartum when sex hormones are reduced and an increase in the frequency of exacerbations is often observed [Korn-Lubetzki *et al*. (1984); Confavreux *et al*. (1998); Ostensen & Husby (1983)]. These findings have led to the hypothesis that hormones, in particular oestrogen may affect regulatory T cells [Offner *et al*. (2000)]. Studies have shown oestrogen can bind to and regulate $CD4^+$ T cells and decrease inflammation by inhibiting Th1 cells or by promoting regulatory Th2 [Wegmann *et al*. (1993)] or $CD8^+$ cells [Stimson (1998)]. Support for this theory comes from studies in which castrated female mice have been found to show acceleration in the onset of EAE.

Testosterone has been implicated in MS severity. It has been found that women with MS tend to have abnormally low testosterone levels compared to disease-free women [Tomassini *et al*. (2005)]. In addition, women with low testosterone levels suffered a greater degree of brain inflammation than those with normal hormone levels. However, women suffering from MS who had abnormally high levels of testosterone were more likely to have a higher incidence of irreversible brain tissue damage [Tomassini *et al*. (2005)].

1.2.4 NOTCH1 pathway

Studies have been performed to investigate the potential reinstatement of the NOTCH1 pathway in MS. During development of the nervous system NOTCH1 is activated by the ligand Jagged1 which in turn activates HES5 resulting in the inhibition of the maturation of oligodendrocytes. John *et al.* (2002) found that *in vitro,* TGFβ1 which is up-regulated in MS has the ability to reintroduce Jagged1 in human astrocytes. It was also found that around MS lesions in which there was little or no remyelination there were high levels of jagged1 and in lesions in which there was remyelination there were very low levels of jagged1. These findings implement the NOTCH1 pathway in the remyelination process in MS and offer a potential for new therapies.

1.3 Pathological mechanisms

Triggers thought to be involved in MS are relatively well defined compared to the understanding of what happens following disease initiation. The control of the disease state, in particular the mechanisms involved in

relapse and remission are far from clear and once again contradictory results have hindered progress.

1.3.1 Adhesion molecules and chemoattractants

There are two categories of molecules that direct leukocyte migration into inflammatory sites; adhesion molecules and chemoattractants. Among chemoattractants, chemokines have been of particular interest, due to their potential role in inflammation [Luster (1998); Rollins (1997); Baggiolini (1998)]. Chemokines selectively attract leukocyte subsets; some chemokines act specifically on neutrophils, others on monocytes, dendritic cells or T cells [Rollins (1997)]. Chemokines appear to act in at least two ways, firstly through direct chemoattraction and secondly by activating leukocyte integrins to bind their adhesion receptors on endothelial cells [Baggiolini (1998); Campbell *et al*. (1998)].

The adhesion molecules P and E selectin are essential for the localisation of T cells to other tissues [Springer (1994)], however they are not required for the development of EAE in animal models [Engelhardt *et al*. (1997)]. This research has been supported by Döring *et al.* (2007) who found by using E/P selectin double-deficient knockout SJL mice that E and P selectins were not required for the recruitment of inflammatory cells into the CNS and for the development of EAE. The knockout mice displayed comparable EAE to that of the wild-type mice. Although these findings would suggest that E and P selectin are not critical for blood brain barrier inflammatory infiltration other studies have found E and P selectin to be expressed in the superficial blood vessels in the brain during EAE which

mediate leuckocyte recruitment [Piccio *et al*. (2002)]. It has been suggested that the role of E and P selectins is species/strain dependent [Carrithers *et al.* (2002)]. Due to the presence of E and P selectin expression in EAE it can not be ruled out that these molecules do not have some role in the development of this disease.

Studies using transgenic mice that over-express chemokines under the control of tissue-specific promotors, indicate that chemokines are sufficient to direct the migration of target leukocytes into the CNS [Tani and Ransohoft (1994); Bell *et al*. (1996); Fuentes *et al*. (1995)], and that vigorous CNS chemokine expression during EAE has a consistent relationship to clinical disease activity [Karpus *et al*. (1995); Karpu & Kennedy (1997); Berman *et al*. (1996); Godiska *et al*. (1995); Glabinski *et al*. (1996, 1997); Hulkower *et al*. (1993); Miyagishi *et al*. (1997); Tani and Ransohoft (1994)].

Production of chemokines by leukocytes and resident CNS cells near inflamed vessels (perivascular cuffing) has been previously described in EAE and reported in MS [Glabinski *et al*. (1997); Miyagishi *et al*. (1997); Hvas *et al*. (1997); McManus *et al*. (1998); Simpson *et al*. (1998)]. It was found that the CXCR3 IP-10Mig receptor, a selective attractant for activated memory T cells, was the predominant chemokine receptor on lymphocytic cells in both CSF and parenchymal compartments, where it was found on 80% of $CD4^+$ cells [Anderson *et al*. (2000)]. CXCR3 positive cells have also been found in perivascular cuffs in MS brain sections

[Sørensen *et al*. (1999)]. It was proposed that activated T cells expressing CXCR3 are preferentially able to gain access to CNS tissue and initiate an autoimmune response [Torben *et al*. (1999); Williams *et al*. (1994)] and thus are likely to have a pathogenic role.

1.3.2 Cytokines

Cytokines are also thought to play a pro-inflammatory and regulatory role in MS [Lin *et al*. (1995); Chitnis *et al.* (2001)] and studies investigating their role may be interpreted in such a way that some cytokines are considered to be the primary pathogenic agents in MS, leaving little place for the antibody theories, unless of course the antibodies themselves act as stimulators of cytokines.

In mammals, Th1 cells secrete IL-12 and IFN-γ and mediate delayed type hypersensitivity, whereas Th2 cells secrete predominantly IL-4, IL-5 and IL-10 and mediate humoral immunity [Mosmann *et al*. (1986)]. Cells that secrete predominantly TGF-β have been termed Th3 cells [Chen *et al*. (1994); Weiner (1997)]. In the EAE model in the mouse, T cells producing Th1 cytokines can transfer disease [Ando *et al*. (1989); Racke *et al*. (1994); Powell *et al*. (1990); Begolka *et al*. (1998)], while spontaneous recovery from EAE correlates with a switch to TGF-β and Th2 cytokines [Khoury *et al*. (1992); Kennedy *et al*. (1992); Issazadeh *et al*. (1995, 1998); Chen *et al*. (1998)]. Other studies have found that Th2-like cells are not always efficient in regulating the effector activity of differentiated autoimmune Th1 effector cells and in some cases may themselves be

capable of causing CNS pathology [Khortus *et al*. (1995); Lafaille *et al*. (1997)]. Despite such contradictions, it has been postulated that a cytokine switch from a pro-inflammatory Th1 to an anti-inflammatory Th2 environment in the CNS immediately precedes or coincides with remission from an acute episode of disease. This is supported by studies that have shown a cyclical expression of mRNA in the CNS encoding Th1 and Th2 cytokines during relapsing-remitting disease [Kennedy *et al*. (1992); Tanuma *et al*. (1997); Racke *et al*. (1991, 1992); Rott *et al*. (1994); Johns *et al*. (1991)]. Recovery from disease in acute self-limiting EAE in Lewis rats and PL/J mice depends on the presence of regulatory T cells that are apparently able to down-regulate ongoing inflammatory autoimmune responses [Sun *et al*. (1988); Lider *et al*. (1989)]. $CD4^+$ regulatory T cells from Lewis rats recovered from acute EAE produce TGF-β to inhibit IFN-γ and TNF-β production [Karpus & Swanborg (1991); Stevens *et al*. (1994)].

IL-12 is produced mainly by monocytes/macrophages in response to bacterial products and intracellular parasites, some B cells and murine dendritic cells also produce IL-12. IL-12 in MS is considered to have a detrimental role, with elevated serum levels and an increase in T cell receptor mediated IL-12 secretion found in the chronic progressive form of MS [Balashov *et al*. (1997); Nicoletti *et al*. (1996)]. Elevated levels of IL-12 p40 mRNA has also been found in the brain and the lymphoid organs of animals in the acute phase of the disease [Issazadeh *et al*. (1995)]. Other studies [Yoshimoto *et al*. (1998)] have found that IL-12 p40 has an antagonist effect on IL-12 and has a possible therapeutic effect by altering

the Th1 environment. IL-18 works in synergy with IL-12 [Ahn *et al*. (1997)] and in humans it can enhance Th1 differentiation [Micalef *et al*. (1996)]. TNF-α also plays a pivotal role in the initiation and progression of the autoimmune inflammatory process [Wildbaum *et al*. (2000)].

Activated T cells, B cells, macrophages, endothelial cells and fibroblasts produce IL-6 [Taga & Kishimoto (1997)]. An elevation of IL-6 gene expression in the brain appears to correlate with the severity of disease [Diab *et al*. (1997); Kennedy *et al*. (1992); Okuda *et al.* (1995)]. It is suggested that IL-6 is important in the induction of disease and/or as a factor necessary for pathogenic T cells to survive.

IL-3 is released from activated T cells, but functions mainly as a growth factor for hematopoietic stem cells. When over expressed in the brains of mice by transgenic technology using the GFAP promotor [Chiang *et al*. (1996); Powell *et al*. (1999)], macrophage/microglial mediated primary demyelination occurs, thus demonstrating the potential toxicity of this cytokine within the CNS, which may arise in the vicinity of active inflammation. In these mice the disease is manifested as chronic progressive rather than relapsing/remitting.

IL-15 has been associated with Th1 and Th2 differentiation with IL-15 causing chronic stimulation of Th1 leading to an autoimmune state. In MS, IL-15 mRNA expression has been correlated with the manifestations of clinical disease in the CNS of patients [Kivisakk *et al*. (1998)].

In rats IL-1 and IL-1Ra (interleukin 1 receptor antagonist), respectively exacerbate and ameliorate clinical disease scores [Jacobs *et al*. (1991)]. This is also true in multiple sclerosis patients where disease progression is controlled in part by the regulation of IL-1 by the IL-1 receptor antagonists (type I and II) and IL-1 accessory protein [Libra *et al*. (2006)]. De Jong *et al*. (2002) found that families having a high IL-1β production compared to IL-Ra production were 2.2 times more likely to have a patient relative with relapsing MS than those families with a low IL-1β:IL-1Ra ratio. Oligodendrocyte destruction in MS may be due in part to interleukin1β (a microglia-derived cytokine), due to its ability to impair the uptake and metabolism of glutamate by astrocytes. A similar effect was seen for TNFα [Takahashi *et al*. (2003)].

IFN-γ is secreted by activated T cells and macrophages. It promotes T cell growth and inhibits IL-4 mediated B cell growth and murine IgG_1 and IgE secretion. In humans, systemic IFN-γ exacerbates MS [Panitch & Bever (1993)]. It is possible that secretion of IFN-γ is necessary to establish the conditions, which subsequently regulate the progress of inflammation and in its absence more tissue damage results, because the inflammatory process continues for longer [Bettelli & Nicholson (2000)].

IL-4 is produced by the Th2 subset of activated T cells, mast cells, NK cells and basophils. It regulates immunoglobulin isotype switching in B cells, stimulating IgG_1 and IgE production [Kuhn *et al*. (1991)]. It drives T cells to differentiate towards the Th2 phenotype [Seder *et al*. (1992);

Swain *et al*. (1990)] and has been shown to reduce the severity of EAE [Racke *et al.* (1994)], especially during the disease induction phase.

IL-10 is produced by Th2 and Th0 $CD4^+$ T cells clones, but not by Th1 or $CD8^+$ T cells, although it has been reported that some $CD8^+$ T cells can secrete IL-10 [Racke *et al*. (1994)]. T cells following stimulation with antigen or polyclonal activators, mouse B cells (especially the Ly1 B cell subset) and CNS resident astrocytes and microglia produce IL-10. The major source of IL-10 is from activated macrophages. IL-10 appears to have a protective role in MS. It has been shown to inhibit the expression of MHC class II antigens and prolonged treatment with IL-10 has been found to inhibit EAE development in SJL mice. SLJ mice are a strain of mouse that are immunocompetent, but have elevated levels of circulating T cells and are frequently used as an EAE model. Studies have also reported IL-10 mRNA peaks before, or during remission in EAE [Issazadeh *et al*. (1995); Kennedy *et al*. (1992)], although other studies have shown an almost constant expression of IL-10 mRNA in the brains of animals with EAE [Begolka *et al*. (1998)].

Many different cell types including lymphocytes and macrophages produce TGF-β. TGF-β has been shown to inhibit proliferation of T and B lymphocytes [Kehrl *et al*. (1986)] and inhibit the effect or production of IFN-γ, TNF-α, TNF-β and IL-2 [Espevik *et al*. (1987)]. TGF-β has a chemotactic effect and induces expression of inflammatory mediators such as IL-1 and TNF-α by monocytes. TGF-β mRNA in the CNS has been

reported to correlate with remission of EAE in the rat model [Issazedeh *et al*. (1996); Tanuma *et al.* (1997)].

A study found that cDNA transcripts for the gene osteopontin were found in MS lesions compared to their complete absence in control brains [Chabas *et al*. (2001)]. In addition to the role of osteopontin in bone maintenance, it also has an immunological role and is found to be expressed in macrophages, neutrophils, dendritic cells, and T and B cells. Osteopontin promotes cell recruitment to sites of inflammation, acts as an adhesion protein in wound healing, mediates cell activation and cytokine production and regulates apoptosis. In MS the presence of osteopontin within plaques may be due to the body trying to repair itself following inflammatory attack.

1.3.3 Complement

The complement system circulates in the blood and is composed of approximately 30 proteins [Mayer (2009)]. The most abundant of the proteins in this system is C3. It is activated *via* the classical pathway, in that it can be activated in response to antigen-antibody binding or undergo self-activation. The ability to self-activate creates a magnified inflammatory response. Once activated, C3 is cleaved by C3 convertase (C4b2a) into two fragments, C3a and C3b [Mayer (2009)]. C3b acts as an opsonin, increasing phagocytic efficiency and C3a binds to basophils and mast cells resulting in the release of vasoactive substances such as histamine. In the body C3b is quickly inactivated by sialic acid which is found on the surface of cells. Some foreign cells such as bacteria cells

have little or no sialic acid which can cause the complement system to escalate resulting in a prolonged inflammatory attack. In such cases, C3b binds to the Factor B protein forming C3b•Bb, this in turn activates more C3 forming C3b•Bb•C3b, a C5 convertase which initiates the membrane attack complex producing more C3b [Mayer (2009)].

Complement deficiency can lead to infection and autoimmunity. In individuals with SLE, the early complement components such as C2, C3 are often found in low levels and this can have a significant effect on the immune system [Sturfelt & Truedsson (2005)]. Similarly low levels of early complement components are found in other immune complex disorders, including MS [Sturfelt & Truedsson (2005)]. In addition complement has a role in lupus nephritis in which it is fixed by anti-dsDNA antibodies and deposited in the glomeruli causing vasculitis, which damages the blood vessels and leads to impaired renal function.

Complement has been implicated in MS pathology, but whether it is essential for disease development is not yet known. Complement may increase damage to myelin due to its ability to enhance phagocytosis. It may enhance antibody dependent cell-mediated cytotoxicity, mediated by macrophages as seen in EAE models. Complement activation may lead to the release of anaphylatoxins such as histamine, which further increases the inflammatory effect. Complement may shape the adaptive immune response in some way that increases the neuroantigen-specific immune response, this has been demonstrated in EAE models [Dempsy *et*

al. (1996)]. A MOG peptide-induced mouse EAE study found that none of these mechanisms were absolutely required for EAE development [Dempsy *et al*. (1996)].

Studies have found C3 levels to be decreased within the CSF during relapses in MS, but this may be a result of low serum levels which are also associated with MS [Gaillard *et al*. (1993)]. An EAE study in mice [Calida *et al*. (2001)] found that inactivation of complement did not prevent EAE signs and that the severity of the disease was greatest in animals negative for C3. If was also observed that in this particular model (MOG-induced) of EAE inflammatory demyelination was not always associated with local complement deposition.

1.3.4 Antibodies and antigens

Antibodies linked to MS are autoantibodies reactive with myelin basic protein, myelin oligodendrocyte glycoprotein and 2′,3′-cyclic nucleotide 3′-phosphodiesterase [Williamson *et al*. (2001)]. However, anti-MBP and anti-MOG antibodies are frequently found in people with other diseases of the nervous system for which demyelination is not a characteristic, this has led to a questioning of the relevance of using these antibodies as biomarkers for MS [Pittock *et al*. (2007)]. With regards to possible antigens triggering the disease state, animal EAE models have used many different CNS associated antigens. Whole CNS material as used in the Lewis rat model in this project induces an acute disease state with no relapse, and negligible demyelination, whereas PLP, MOG and lipoprotein induce a disease state characterised by inflammation and demyelination.

Other encephalitogenic epitopes are thought to be MAG, GFAP, S-100, and possibly stress proteins [Kojima *et al*. (1994)]. Antigens that have the potential to induce a demyelinating immune response are galactocerebroside [Dubois-Dalq *et al*. (1970)] which is found in nervous tissue components (e.g. myelin), gangliosides GM1 and GM4 [Roth *et al*. (1985); Schwerer *et al*. (1986)] which are found on the cell surface of neural cells (e.g. astrocytes) and sulfatide which is synthesised by oligondendrocytes.

Research has been carried out into finding out if it is the location of a particular antigen that increases the potential to induce MS. One such study [van Noort *et al*. (1995)] investigated the potential of an antigen specific to the white matter in the brains of MS patients to activate T cells. Using reverse phase HPLC it was found that a protein fraction of MBP in the brains of MS patients stimulated the proliferation of T cells taken from MS patients and healthy controls, this was not the case in protein fraction samples taken from the control group. It was found that the heat shock protein alpha-crystallin (CRYAB) was expressed in glial cells of MS lesions. This protein has neuroprotective properties and is a $CD4^+$ T cell target in the myelin sheath of patients with MS. Studies [Ousman *et al*. (2007)] have shown CRYAB to slow down or stop inflammatory pathways. Animal studies using CRYAB knockout mice found that these animals exhibited a more severe type of EAE with higher Th1 and Th17 cytokine secretion from T cells and macrophages than mice positive for CRYAB. The protective effect in the CNS appears in part to be due to the anti-

apoptotic properties of CRYAB. This finding led to the suggestion that by preventing an immune response to CRYAB, i.e. the development of anti-CRYAB antibodies, or by treating with CRYAB itself, symptoms could be prevented or halted.

1.3.5 Anti-double-stranded DNA antibodies

In view of the elusive pathogenic agent in MS, this project has turned the attention on anti-dsDNA antibodies, for which very little research in this area has been reported. As these antibodies are strictly regulated in normal individuals, their presence is often associated with a dysfunctional autoimmune state [Hahn (1998)] and as such potentially of significant pathological importance.

Although antibodies themselves are thought to play a key role in MS pathogenicity they are also part of a vicious circle of escalating immune responses [Hahn (1998)]. A T cell-mediated inflammatory response stimulates apoptosis which is cleaned up by low affinity natural antibodies, typically of the IgM class [Hahn (1998)]. In some cases, the lack of B cell regulation allows these low affinity antibodies to switch into high affinity IgG antibodies that bind to DNA and other autoantigens, resulting in the fixation of complement and an enhanced inflammatory response [Hahn (1998)].

It is suggested that even a single autoreactive H (heavy) Ig chain that escapes tolerance may be enough to induce features of autoimmune disease [Radic *et al*. (1995)]. A tight correlation between lack of allelic

exclusion (where the protein from only one allele is expressed, leading to high affinity antigen-specific antibodies and B cell survival) and anti-dsDNA antibody specificity, is thought to prevent the maintenance of a pool of potentially activatable autoreactive cells [Iliev *et al*. (1994)]. In SLE, it is well recognised that anti-dsDNA antibodies are primarily responsible for the pathogenesis of the disease, with circulating immune complexes depositing in the renal glomeruli, which eventually leads to renal failure. If such antibodies are responsible for damage in SLE then it can be assumed that their presence in cerebrospinal fluid and their increased incidence surrounding plaque formations within the spinal cord of MS patients is not likely to be beneficial.

In addition to SLE, other diseases have shown an association with anti-dsDNA antibodies, although in general to a much lesser extent. One study [Alnaqdy *et al*. (2007)] which used an ELISA, found raised sera levels of anti-dsDNA antibodies in 17% of people with rheumatoid arthritis, 23% of people with mixed connective tissue diseases and in 60% in people with SLE, this was compared to 3% in healthy controls. It is possible that for some diseases anti-dsDNA antibodies may be compartmentalised in the parenchyma or CSF as found in MS [Williamson *et al*. (2001)], away from the circulating blood and as such its association with the disease may be overlooked.

The mechanism behind the pathological role of anti-dsDNA antibodies may not be the same for all diseases and may not be responsible for all

aspects of the disease, particularly in those where multiple organs are affected. In SLE, anti-dsDNA antibodies are primarily responsible for renal disease due to the formation and accumulation of immune complexes in the glomeruli which induce an inflammatory response, leading to tissue scarring and ultimately a loss of renal function [Pisetsky (1998)].

Considerable work has been carried out on anti-dsDNA antibodies in relation to their pathological role in SLE, this is in contrast to the limited amount of published information associated with their suggested role in MS. Much of the information regarding the properties, in particular the pathogenic role of anti-dsDNA antibodies has been obtained from studies using animal models of SLE. Although the actual pathological mechanism may be different in MS, much of the following may apply to their role in MS and if nothing else provides a useful starting point.

Splenic anti-ssDNA B cells are short lived, they exhibit an immature, antigen experienced phenotype and localise to the T/B interface of the splenic follicle [Noorchashm *et al*. (1999)]. They are functionally unresponsive to anti-IgM and LPS stimulation and do not phosphorylate intracellular proteins upon mIgM (membrane IgM) cross-linking [Noorchashm *et al*. (1999)]. Anti-dsDNA B cells however phosphorylate intracellular proteins, including Syk upon mIg stimulation and anergy is maintained in the absence of T cells. In response to these signals, anti-dsDNA B cells remain viable, up-regulate cell surface expression of B7.2 (CD86, which has been implicated in allergic inflammation) and IgM and

restore their ability to proliferate and phosphorylate Syk upon mIg cross-linking [Noorchashm *et al*. (1999)].

In SLE, anti-DNA antibodies arise as a result of an antigen driven response [Shlomchik *et al*. (1990); Diamond *et al*. (1992); Radic & Weigert (1994); Tillman *et al*. (1990)] e.g. by bacterial proteins or polysaccharides and peptides that can be molecular mimics of dsDNA. A model for the progression of the anti-DNA antibody response in murine models of SLE suggests that clonal expansion and selection of non-mutated IgM anti-dsDNA antibodies is followed by somatic mutation, which begins before isotype switching to IgG. Further accumulation of mutations has the effect of increasing the affinity for DNA. However, studies in autoimmune MRL/lpr mice have found that such antibodies lacked evidence of somatic mutation, suggesting that affinity maturation *via* somatic mutation is not essential for dsDNA reactivity [Radic *et al*. (1993)]. A general picture of the pathogenic autoantibody that emerges is one of high affinity cationic IgG molecules that bind dsDNA and fix complement resulting in activation leading to an inflammatory response. A study performed by Sontheimer & Gilliam (1978) found that complement fixation by anti-dsDNA antibodies was primarily seen in SLE patients with lupus nephritis and did not occur in those patients for whom nephritis was absent. This finding suggests that the anti-DNA antibodies found in the latter group of patients differed in some way compared to the complement fixing antibodies found in the presence of lupus nephritis.

Ohnishi *et al.* (1994) found that autoantibodies with pathogenic properties had a greater number of positively and negatively charged residues than did the non-pathogenic group of antibodies. In other studies, it has been suggested that pathogenicity might not be related to the charge and affinity of an antibody, but to its fine binding specificity [Putterman *et al*. (1996)]. Some authors have reported the greater potency of anti-nucleosome antibodies as mediators of tissue damage compared with anti-DNA antibodies [Burlingame *et al*. (1994)] and other studies have found that anti-ssDNA antibodies can occasionally be more damaging than antibodies that bind to dsDNA [Swanson *et al*. (1996)]. The relationship between specificity of binding and the degree of mutation does not apply to all anti-DNA antibodies. It is quite possible for a natural polyreactive IgM antibody, to contain many somatic mutations or a specific IgG and dsDNA antibody to contain few mutations [Rahman *et al*. (1998)].

Identification of the antigen responsible for raising pathogenic DNA antibodies has proved to be less obvious than one would think. DNA, even in the presence of an adjuvant, is not immunogenic *in vivo*, supporting the theory that DNA complexed to histones in the form of nucleosomes is the key immunogenic particle responsible for the production of anti-DNA antibodies [Moens *et al*. (1995)]. Nucleosomes are the fundamental repeating units of chromatin and are released from the nucleus during apoptosis by inter-nucleosomal cleavage of the chromatin [Schwartz & Osborne (1993)]. Apoptosis leads to the development of blebs that contain nuclear material on the cell surface, suggesting that

under some circumstances apoptosis could be an essential step in the development of anti-dsDNA antibodies [Rose *et al*. (1997)]. This implies that interference with apoptosis may be important in the pathogenesis of autoimmune disease.

In non-SLE susceptible mice, bacterial DNA complexed to methylated bovine serum albumin in a suitable adjuvant can induce the production of high titres of IgG anti-DNA antibodies [Gilkeson *et al*. (1991)]. Anti-DNA antibodies induced in this way do not bind to mammalian dsDNA, they do however, react with mammalian histone proteins complexed with mammalian RNA. This provides evidence for the potential induction of autoimmune anti-dsDNA antibodies following bacterial infection. There are several mammalian histone peptides and other components that are common target antigens in autoimmune disease (typically mixed connective tissue diseases), for example Sm-D antigens (e.g. SLE), ubiquitinated histone H2A antigen (e.g. SLE) and poly (ADP-ribose) antigens (e.g. rheumatoid arthritis) [Atanassov *et al*. (1991)]. Protein antigens may also be responsible for loss of tolerance to dsDNA in systemic disease [Putterman *et al*. (2000)].

In SLE mouse models, the onset of the autoimmune response is characterised by the early emergence of antibodies that recognise conformational epitopes of the nucleosome particles [Burlingame *et al*. (1993); Amoura *et al*. (1994)]. As the immune response progresses, autoantibodies that react to dsDNA or histones become apparent.

Nucleosomes have been found to be a major immunogen for inducing Th cells that are able to stimulate pathogenic autoantibodies in mice with lupus [Mohan *et al*. (1993)]. In a study where human hybridoma-derived monoclonal anti-DNA antibodies were produced (IgG and IgM), two of the IgG antibodies bound both to dsDNA and histones, whereas none of the IgM antibodies did. The binding of these human monoclonal antibodies to histones was enhanced by the presence of DNA [Ehrenstein *et al*. (1993)].

Epitope spreading may be a mechanism by which anti-DNA antibodies are generated [James. (1991)]. Activated phagocytic cells such as polymorphonuclear leukocytes and macrophages are capable of releasing highly reactive oxygen species which may be able to penetrate cell membranes and interact with and damage nuclear DNA. Subsequent release of the altered DNA may then stimulate the production of anti-DNA antibodies by B cells.

Studies have found that in SLE, anti-DNA antibodies can induce IFN-α production when combined with the appropriate kind of DNA. It was suggested that the various immunomodulatory actions of type 1 IFN (IFNα/β), including the promotion of expression of the IL-12 receptor and survival of activated T cells, could counteract the maintenance of self-tolerance [De Maeyer & De Maeyer-Guignard (1988); Belardelli (1995); Rogge *et al*. (1997, 1998); Marrack *et al*. (1999); Tough & Sprent (1998)]. An increased rate of apoptosis caused in T cells by IL-10 for example [Georgescu *et al*. (1997)] and in virus infected cells by type 1 IFN [Tanaka

et al. (1998)] may generate relevant nuclear autoantigens and possibly endogenous dsDNA, the latter forming IFN-α inducing complexes with anti-DNA antibodies. A mechanism resembling a vicious cycle may sustain the disease process by means of continuous IFN-α production, occasionally boosted by infections or other processes that generate more autoantibodies, costimulatory cytokines and DNA [Vallin *et al*. (1999)].

It has been demonstrated that native mammalian DNA complexed with an immunogenic DNA binding peptide Fus1 from *Tyrpanosoma cruzi* can induce anti-DNA antibodies in mice not genetically prone to autoimmune disease. Although of a lower titre, the induced anti-DNA antibodies were found to be of similar specificity, structure and immunopathological function as that seen for autoimmune anti-DNA antibodies and resulted in the development of lupus nephritis [Desai & Marion (2000)]. Further more it was found that DNA-Fus1-induced anti-DNA and anti-Fus1 antibodies levels were dependent on the presence of the H-2 haplotype. Animals with the H-2^z haplotype had the highest titres, which was to be expected in light of the link found between H-2^z and autoimmune anti-DNA antibody production [Desai & Marion (2000)]. In a different study, pre-autoimmune NZB/W mice (a strain of mouse known to produce high affinity anti-dsDNA antibodies and glomerulonephritis) immunised with *Escherichia coli* dsDNA produced antibodies that resemble spontaneous autoantibodies that bind to mammalian dsDNA. However, when normal mice were similarly immunised, they produce antibodies that only bind to bacterial dsDNA [Wloch *et al*. (1997)]. These findings are also of relevance to anti-

dsDNA antibody production in MS, in particular with regards to the association with genetic make-up, with the presence of the MHC haplotype being important in MS susceptibility.

Proposed mechanisms of T cell help for anti-dsDNA antibody secretion include T cell recognition of DNA associated protein antigens such as histones and recognition of anti-DNA antibody-derived peptides in the context of class II MHC [Hardin & Thomas (1983); Mohan *et al*. (1993); Ebing *et al*. (1993); Singh *et al*. (1995)]. The presentation of such antigens could initiate T cell activation to these self components which in turn would help B cells to secrete antibodies specific to histones of dsDNA for example, thus producing an autoimmune response. It has been suggested that DNA alone is unlikely to be a T cell epitope and that carrier proteins e.g. histones, bound to DNA may be involved in creating its antigenicity [Lu *et al*. (2003)]. If this was to be the case then auto-reactive T cells that recognise these carrier proteins would provide help to anti-dsDNA antibody secreting B cells. B cells have the ability to recognise haptens, which in this example is dsDNA, as well as carrier proteins. With the loss of tolerance, B cells would secrete anti-dsDNA antibodies and antibodies to the carrier proteins [Lu *et al*. (2003)].

The potential for anti-dsDNA antibodies to be pathological is supported by a significant amount of research. In general, anti-dsDNA antibodies are not found in healthy individuals, but are often seen in individuals with automimmune diseases [Hahn (1998)]. They have been proven to have a

pathological role in SLE, in particular in relation to lupus nephritis [Pisetsky (1998)]. The existence of anti-dsDNA antibodies fits into the molecular mimicry and/or epitope spreading theories.

Anti-ds-DNA antibodies have been found to have a number of pathogenic features such as their potential to induce IFNα and affect apoptosis. With regards to MS, evidence of anti-dsDNA antibodies being a major component of CSF and their presence on the outer edge of active lesions [Williamson *et al*. (2001)] makes them prime suspects in the disease process. Williamson *et al* proposed that these antibodies are associated with an autoimmune state resulting from impaired B cell regulation leading to the generation of anti-dsDNA antibodies, which precede disease development. This has been found to be the case in a Russian study [Ershova *et al*. (2003)] of MS patients where an association between increased anti-dsDNA antibody titre and the level of disability was observed.

1.4 Animal models

Animal models of MS are important for the understanding of the pathology and pathogenesis of the disease in humans and as such for the development of new therapies to control disease progression. Animal models are vital in MS research due to the target organ being the CNS, which makes research on human tissue from patients living with MS extremely difficult, if at all possible. However, due to the complexity of MS and the different progressions it can take, there is no one animal model

that mimics the disease in its entirety and as such researchers select the model most appropriate to the question they are trying to answer.

Animal models of MS are typically known as experimental allergic (or autoimmune) encephalomyelitis models and depending on the immunisation agent, dosing regime and species/strain of animal used can be used to investigate the mechanisms involved in causing inflammation and/or demyelination/remyelination and also the efficacy of new treatments.

1.4.1 Models of demyelination

Demyelination/remyelination processes can be studied in a number of different animals, for example MAG knockout mice [Lunn *et al.* (2002)] or myelin mutant animals such as the myelin basic protein mutant Taiep rat [Foote & Blakemore (2005)] or PLP mutant Rumpshaker and Jimpy mice [Yool *et al.* (2000)].

The Taiep rat develops chronic demyelination with age and by the time it is one year old, demyelination in this rat is considered to have many similarities to that observed in MS patients [Foote & Blakemore (2005)]. In this model demyelination occurs in the absence of inflammation [Foote & Blakemore (2005)].

The Jimpy mouse displays severe neurological dysfunction due to the reduced ability of the CNS to form myelin. These animals display clinical

signs of neurodegeneration such as tremor and convulsion, often resulting in a premature death [Yool *et al.* (2000)].

The Rumpshaker mouse has a less severe phenotype compared to the Jimpy mouse and in general can survive into adulthood [Yool *et al.* (2000)].

1.4.2 Viral models

Viral EAE models involve the immunisation of an animal with a virus that is known to target the CNS. One such virus is Theiler's murine encephalitomyelitis virus which is considered by some researchers to result in one of the best animal models of MS presently available [Dal Canto *et al*. (1995)]. Using genetically susceptible mice such as SJL mice, this virus can induce paralysis, CNS inflammation and demyelination [Dal Canto *et al*. (1995)]. Research with this particular model has shown how viral and bacterial infection can induce demyelinating disease by molecular mimicry [Miller *et al*. (1997)].

1.4.3 CNS material-induced EAE

This model is frequently used by researchers. The methodology is relatively straight forward and in the right hands is reproducible.

EAE in this model has been induced by whole spinal cord homogenate [Levine and Sowinski (1980 &1989)], MBP [Asensio *et al*. (1999)], MOG [Reynolds *et al*. (2002)] and PLP [Chalk *et al.* (1994)] to name a few agents. Typically the encephalitogenic agent is combined with an

adjuvant e.g. carbonyl iron [Levine and Sowinski (1980 &1989)] or Freund's complete adjuvant [Asensio *et al*. (1999)]. Immunisation regimes such as a single intraperitoneal injection [Levine and Sowinski (1980 &1989)] or by injection into the base of the tail [Heremans *et al.* (1999)], subcutaneous injection into the flank [Liblau *et a*l. (1997)] or into the foot pad [Delarasse *et al*. (2003)] followed approximately a week later by a booster injection have been used to induce EAE.

In the majority of cases, for EAE induction to be successful autoimmune susceptible animals, in particularly those that display a strong Th1 response to immunisation are required. Strains found to be susceptible to EAE are the Lewis rat, Biozzi (antibody high) mouse, the SJL mouse and the C57BL/6 mouse. The progression of EAE depends on the encephalitogenic agent and the strain of animal used. For example, Biozzi (ABH) and SJL mice develop a relapsing/remitting disease when immunised with whole myelin [Lavi & Constantinescu (2005)]. C57BL/6 mice on the other hand are EAE resistant when immunised with whole myelin [Lavi & Constantinescu (2005)], but when immunised with MOG these mice develop a chronic progress type of EAE. The Lewis rat develops acute EAE following immunisation with guinea-pig whole spinal cord [Levine and Sowinski (1980 &1989)].

Following immunisation with the encephalitogenic agent the animals develop a limp tail and in some cases paralysis of the hind limbs [Beeton *et al*. (2007)]. In the case of a relapsing/remitting or acute model signs are

first apparent approximately 9 - 14 days following immunisation and disappear within approximately 5 days of the emergence of the first signs. In acute models no further signs are seen. In relapsing/remitting models approximately 2 weeks later signs reappear. With each episode the severity of signs may increase and remission may not be complete [Beeton *et al*. (2007)]. In the chronic progressive model the animals enter a continuous decline leading to paralysis and death within a few weeks [Lavi & Constantinescu (2005)]. The actual disease profile depends upon the species and encephalitogenic agent used.

Histopathologically inflammatory infiltrates are present within the CNS and in some models, in particular the relapsing/remitting models of EAE demyelination is present.

In this project the acute EAE Lewis rat and the relapsing/remitting Biozzi (ABH) mouse EAE models were selected. The Lewis rat was selected as this model is good for investigating the inflammatory aspects of the disease. The Biozzi (ABH) mouse was used as this mouse exhibits a relapsing/remitting form of the disease with increasing levels of autoantibodies with each subsequent EAE episode.

1.4.4 Spontaneous EAE models

There are no known spontaneous animal models of MS. However, transgenic mice which have myelin-specific T cell receptors in all of their T cells spontaneously develop an autoimmune state as a result of the slightest antigenic trigger [Lavi & Constantinescu (2005)]. The problem

with this particular model is that the C57BL/6 mouse which is often used for this purpose exhibits a rapid and chronic progression of EAE, which may make research using these particular animals difficult [Lavi & Constantinescu (2005)]. Transgenic animal models have helped researchers understand the role of the MHC [Friese *et al.* (2006)].

1.4.5 Non-human primates in MS research

There are instances in drug development when rodents are not an appropriate species for the testing of a new drug. Such instances may arise if the drug is a human monoclonal antibody with high receptor specificity which would be ineffective in rodents. In these instances the use of a higher species such as a non-human primate for research purposes may be justified.

For MS research the non-human primate used is the common marmoset [Hart *et al*. (2005)]. The marmoset has many immunological similarities with humans and is highly susceptible to developing EAE. In contrast to the rodent models of EAE, where the animals are frequently killed and examined histologically for EAE effects, the disease process (CNS lesions) is often visualised in the primate using non-invasive magnetic resonance imaging techniques [Hart *et al*. (2005)]. In the study performed by Hart *et al*., EAE was induced by a single immunization with recombinant human myelin/oligodendrocyte glycoprotein. Active CNS lesions in this model remain active for a prolonged time [Hart *et al*. (2005)].

1.5 Project overview

The aim of this project was to investigate the potential pathogenic role of anti-dsDNA antibodies in multiple sclerosis. In order to do this the project set out to:

- Clinically and histopathologically profile the EAE model in the Lewis rat.
- Using the profile information determine the most likely time-point during the disease progression when anti-dsDNA antibodies would most likely be present.
- Develop a novel technique for the visualisation of anti-dsDNA antibodies *in situ*.
- Investigate structure function relationships in these antibodies.

2 CHAPTER 2: METHODS

2.1 The Profiling of Parameters of Interest in Experimental Allergic Encephalomyelitis in the Female Lewis Rat; an Acute Model of EAE - in vivo phase

2.1.1 Introduction

This chapter details the methodology used to obtain sera, cerebrospinal fluid, spinal cord and brain samples from rats which had been subjected to experimental allergic encephalomyelitis. Samples obtained in this test were used to validate methods for the detection of anti-ssDNA and anti-dsDNA antibodies in sera and cerebrospinal fluid using an enzyme-linked immunosorbant assay and immuno-staining techniques for the detection of deposits of IgG, IgM, complement (C3) and B cells in spinal cord and brain sections. In addition, these techniques were used to profile acute experimental allergic encephalomyelitis progression in the female Lewis rat.

Acute EAE in the Lewis rat has been previously described by Levine and Sowinski (1980 &1989).

2.1.2 Materials

Sixty-eight female Lewis rats from different litters were obtained from Charles River (UK) Ltd., Kent, for use in this study. The rats weighed between 101 and 139 g on their respective day of dosing (Day 1), and were approximately 6 weeks of age. Six male Dunkin-Hartley guinea pigs

(weighing approximately 600 g) were supplied by David Hall, Staffordshire, UK for the extraction of central nervous system material which was used for the induction of EAE. The adjuvant and vehicle control was carbonyl iron (batch 79H1139), supplied by Sigma, UK which was formulated in sodium chloride (saline: batch 6230), supplied by Fresenius Ltd., UK.

2.1.3 Preparation of materials

The encephalitogenic agent (whole spinal cord) was obtained from six healthy male guinea pigs that had been previously shaved in order to minimise contamination of the spinal cord with fur. Following euthanasia by an intraperitoneal overdose of sodium pentobarbitone, the entire spinal column was removed (from the pelvic region to the high cervic) and cut into three pieces. Each section of spinal cord was exposed by carefully cutting away the vertebrae from the inner aspect of the spinal column. Once exposed, the spinal cord, complete with myelin sheath was teased out and placed in a petri dish. A total of 4.5 g of spinal cord was used in this part of the project.

The encephalitogenic agent was prepared by homogenising 4.5 g of spinal cord with 4.5 g of carbonyl iron in 45 mL of 0.9% (w/v) saline to give a final concentration of 100 mg/mL (CNS material):100 mg/mL (adjuvant).

The vehicle control (carbonyl iron) was formulated in 0.9% (w/v) saline at a concentration of 100 mg/mL.

All formulations were stored at room temperature (nominally 15 to 25°C) in sealed containers and protected from light prior to dosing.

2.1.4 Environment

The rats were housed in groups of four in polypropylene cages (approximately 45 x 28 x 20 cm) with solid floors and Grade 10 woodflakes, supplied by Datesand Ltd., UK as bedding. The cages were cleaned and dried before use. Aspen chew blocks, supplied by Datesand Ltd., UK were placed within the cages as a form of environmental enrichment.

Holding rooms were maintained within acceptable limits for temperature and relative humidity (19 to 25°C and 40 to 70 %, respectively). The room was illuminated by fluorescent light for 12 hours (6am – 6pm) out of each 24 hour cycle and designed to receive at least 15 fresh air changes per hour.

2.1.5 Diet and water

Diet (RM1.(E).SQC., Special Diets Services Ltd., UK) and water from the mains tap supply were provided *ad libitum*. These are routinely analysed for specific constituents and are not known to contain any biological or chemical entity, which might interfere with the test system.

2.1.6 Animal health and welfare

All procedures to be carried out on live animals as part of this study were subject to the provisions of United Kingdom Law, in particular the Animals (Scientific Procedures) Act, 1986.

2.1.7 Acclimatisation and health procedures

On arrival, all animals were examined for ill-health. Animals were acclimatised for a period of at least 3 days.

2.1.8 Random allocation to treatment groups

Prior to the start of the study, animals were allocated randomly to treatment groups as they came to hand (Tables 1 and 2) and individually tail-marked with a number using an indelible pen.

2.1.9 Experimental design

2.1.9.1 Part I

Food and water were available at all times, except when the animals were removed from their home cage for the study procedures.

On Day 1, animals in Group 2 (a-d) received a single 1 mL intraperitoneal injection of CNS material (100 mg):carbonyl iron (100 mg). Animals in Group 3 (a-d) received a single 1 mL intraperitoneal injection of carbonyl iron (100 mg/mL) and served as a control group. Animals in Group 1 remained untreated.

Treatment groups (2a-d) for which signs of EAE were expected, consisted of eight rats per group. A total of eight rats per group was considered to be a sufficiently high enough number to allow for the presence of non-responders and at the same time keeping animal usage to a minimum. For the control groups (1, 3a-d) for which no signs were expected, group sizes of four were used, thus minimising animal usage, without compromising the objective of this study. This rationale was based on previous experience with this model.

Group	Treatment	Point of sampling	Number of animals
1	Untreated	Beginning and end of observation period	4
2a	CNS:carbonyl iron (100 mg/mL:100 mg/mL)	Onset of EAE	8
2b	CNS:carbonyl iron (100 mg/mL:100 mg/mL)	Plateau of EAE	8
2c	CNS:carbonyl iron (100 mg/mL:100 mg/mL)	Remission of EAE	8
2d	CNS:carbonyl iron (100 mg/mL:100 mg/mL)	Day 34 ('relapse')	8
3a	Carbonyl iron (100 mg/mL)	For every 2 Group 2a animals 1 animal from this group was sampled	4
3b	Carbonyl iron (100 mg/mL)	For every 2 Group 2b animals 1 animal from this group was sampled	4
3c	Carbonyl iron (100 mg/mL)	For every 2 Group 2c animals 1 animal from this group was sampled	4
3d	Carbonyl iron (100 mg/mL)	Day 34 ('relapse')	4

Table 1 Treatment groups for Part I of the Lewis EAE *in vivo* phase

The rats were observed daily from Day 1 to Day 34 (as appropriate) and scored for signs of EAE as follows:

0 Normal

1 Slight limpness of tail (distal half)

2 Moderate limpness of the tail

3 Substantial limpness of tail (entire tail)

4 Ataxia/hind limb weakness

5 Extreme weakness of hind limbs, but not paralysis (response to toe pinch)

6 Hind limb paralysis (dragging hind limbs behind when moving)

It is possible for any one animal to have more than one of the scores shown above and in such instances the scores are added together. For example, an animal displaying a substantially limp tail and an ataxic gait would have a total score of 7.

The scoring system used was developed on the basis of previous experience with this model.

Bodyweights were recorded twice weekly from Day 1 to Day 34, as appropriate.

2.1.9.2 Part II

Food and water were available at all times, except when the animals were removed from their home cage for study procedures

On Day 1, animals in Group 5 received a single 1 mL intraperitoneal injection of CNS material:carbonyl iron (1:1 ratio). Animals in Group 6

received a single 1 mL intraperitoneal injection of carbonyl iron (100 mg/mL).

Animals in Group 4 remained untreated.

Rationale for group sizes was the same as stated previously.

Group	Treatment	Point of sampling	Number of animals
4	Untreated	Pre-dose	4
5	CNS:carbonyl iron (100 mg/mL:100 mg/mL)	Peak EAE	8
6	Carbonyl iron (100 mg/mL)	For every 2 Group 5 animals 1 animal from this group was sampled	4

Table 2 Treatment groups for Part II of the Lewis EAE *in vivo* phase

Rats in Groups 5 and 6 were observed daily from Day 1 until they were sampled at the time of peak EAE signs for Group 5, or as appropriate for Group 6. EAE was scored as detailed previously.

Bodyweights were recorded on Day 1, 5, 8, 12 and 15, as appropriate.

2.1.9.3 Sampling time criteria

Blood, cerebrospinal fluid, spinal cord and brain were collected from each animal at the appropriate time, as shown in Tables 1 and 2. Two untreated animals from Group 1 were sampled on Day 4, with the remaining two rats sampled on Day 34. In Part II, two untreated animals from Group 4 were sampled on Day 4, with the remaining two rats

sampled on Day 15. This was done to allow for age differences between the animals from the start to the end of the study.

Using a self-set criterion, onset was taken as the time at which the first detectable signs of EAE were observed, this being a slight limpness of the distal part of the tail. Peak was taken as the time at which the first marked signs of EAE were observed, this being at least a substantially limp tail. Plateau was taken as the time at which the same signs of comparable severity had been observed on at least two consecutive days. Remission was taken as the first day that the animals appeared normal. Relapse was not seen in this study, the animals were therefore killed and sampled on Day 34 in order to see if there had been any histological relapse.

2.1.9.4 Sampling procedures

Terminal blood samples (approximately 2 mL) were taken from anaesthetised (sodium pentobarbitone administered intraperitoneally) animals *via* cardiac puncture and collected in plain tubes. Blood samples were allowed to clot for 2–4 hours at room temperature (nominally 15 to 25°C) and then overnight in the refrigerator (nominally 2 to 8°C). The blood was centrifuged at approximately 3000 rpm for 10 minutes at 4°C and the serum harvested and divided into two aliquots. Sera was stored frozen (nominally –70°C) prior to antibody analysis.

Immediately following blood sampling, the rats were killed by an intravenous overdose of sodium pentobarbitone and approximately 0.1 mL

of CSF was collected into plain tubes. CSF samples were collected by inserting a butterfly needle (23 gauge, 0.75 inch, supplied by BD Infusion Therapy, Sweden) into the cerebellomedullary cistern. Correct insertion of the needle was confirmed by the presence of straw coloured CSF flowing into the cannula. Care was taken not to contaminate the CSF with blood. CSF was stored frozen (nominally -70°C) prior to antibody analysis.

The brain was removed and cut into three sections (cerebellum, parietal lobe and frontal lobe) and flash frozen in iso-pentane cooled in liquid nitrogen. Brain samples were stored at -70°C.

The entire spinal cord was removed from the inner aspect, by carefully cutting away the vertebrae. The cord was teased from the cavity and cut transversally in half. Both sections were flash frozen in iso-pentane cooled in liquid nitrogen. Spinal cord samples were stored at -70°C prior to analysis.

2.2 Method Validation of Experimental Allergic Encephalomyelitis in the Biozzi (ABH) Mouse; a Relapsing/Remitting Model of EAE - in vivo phase

2.2.1 Introduction

Due to the limited success in detecting anti-dsDNA antibodies in the rat, an attempt was made to validate an EAE model, which was expected to have a relapsing/remitting profile and a model in which, unlike acute EAE in the rat features demyelination, which is particularly evident in relapse.

By using a relapsing/remitting model it was hoped that with each episode continuing damage would lead to an increase in anti-dsDNA antibody levels, thus increasing the chance of detection.

The Biozzi (ABH) mouse is a well-documented strain of mouse used in the study of immunological diseases and shows high susceptibility in models of EAE [Liblau *et a*l. (1997) and Heremans *et al.* (1999)].

The duration of each validation study in general decreased with each attempt. This was due to awareness that if initial signs had not developed within three weeks they were not likely to do so. The same was true for signs of relapse.

2.2.2 Materials

Sixty-six female and thirty male Biozzi (ABH) mice weighing between 13 and 37 g and approximately 6 to 12 weeks old on their respective Day 1, were obtained from Harlan (UK), Bicester. Male Dunkin-Hartley guinea pigs (approximately 600 g) were supplied by David Hall, Staffordshire, UK for central nervous system harvesting. Biozzi (ABH) mice were supplied by Harlan (UK) for spinal cord donation. CD-1 mice were supplied by Charles River (UK) Ltd., Kent for spinal cord donation. The adjuvants were identified as carbonyl iron (batch 79H1139), Freund's complete adjuvant (batch 11K8928) and Freund's incomplete adjuvant (batch 111K8926) and were supplied by Sigma, UK. *Mycobacterium butyricum* (heat killed), batch not supplied, was supplied by DSMZ, Braunschweig, Germany. Sodium chloride (saline: batch 03K24BD, 03E30BR) was

supplied by Baxter Healthcare Ltd., Norfolk, UK. Bovine myelin basic protein (batch 011K7045) and murine myelin basic protein (batch 014K1341) were supplied by Sigma, UK.

2.2.3 Preparation of materials

Five different encephalitogenic agents were used: Whole spinal cord from healthy untreated guinea-pigs, whole spinal cord from Biozzi (ABH) and CD-1 mice, murine MBP and bovine MBP.

The encephalitogenic agent (whole spinal cord) had been previously obtained from healthy male guinea pigs. Spinal cord from Biozzi (ABH) and CD-1 mice was collected following euthanasia by an intraperitoneal overdose of anaesthetic (sodium pentobarbitone). The entire spinal column was removed (from the pelvic region to the high cervic) and cut into three pieces. A 5 mL syringe was filled with air and held at one end of the spinal column in such a way that a good seal was formed. The syringe plunger was rapidly depressed causing air to blow the spinal cord out of the spinal column.

For Part I, finely chopped Biozzi (ABH) mouse spinal cord was formulated in saline and FCA or FIA at the required concentration of 10 mg/mL. For Part II and IV, homogenised Biozzi (ABH) mouse spinal cord was formulated in saline and FCA or FIA at the required concentration of 10 mg/mL. For Part V, homogenised guinea-pig or CD-1 mouse spinal cord was formulated in saline and FCA or FIA at the required concentration of 10 mg/mL. For Part III, homogenised Biozzi (ABH)

mouse spinal cord was formulated in carbonyl iron at the required concentration of 100 mg/mL CNS material:100 mg/mL carbonyl iron. Bovine and murine MBP were formulated in saline and FCA or FIA at the required concentration of 1 mg/mL. For Part VII and VIII, freeze-dried CD-1 mouse spinal cord was formulated in saline and FCA or FIA at the required concentration of 10 mg/mL and 3 mg/mL of *Mycobacterium butyricum* was added.

All formulations were stored at room temperature (nominally 15 to 25°C) in sealed containers and protected from light prior to dosing.

2.2.4 Environment

The mice were housed in groups of no more than five in polypropylene cages (approximately 33 x 15 x 13 cm) with solid floors and Aspen wood chips, supplied by Datesand Ltd., UK) as bedding. The cages were cleaned and dried before use. Aspen chew blocks, supplied by Datesand Ltd, UK were placed within the cages as a form of environmental enrichment.

Holding rooms were maintained within acceptable limits for temperature and relative humidity (19 to 25°C and 40 to 70 %, respectively). The room was illuminated by fluorescent light for 12 hours (6am – 6pm) out of each 24 hour cycle and designed to receive at least 15 fresh air changes per hour.

2.2.5 Diet and water

Diet (supplied by RM1.(E).SQC., Special Diets Services Ltd., UK) and water from the mains tap supply were provided *ad libitum*. These are routinely analysed for specific constituents and are not known to contain any biological or chemical entity which might interfere with the test system.

2.2.6 Animal health and welfare

All procedures to be carried out on live animals as part of this study were subject to the provisions of United Kingdom Law, in particular the Animals (Scientific Procedures) Act, 1986.

2.2.7 Acclimatisation and health procedures

On arrival, all animals were examined for ill-health. Animals were acclimatised for a period of at least 3 days.

2.2.8 Random allocation to treatment groups

Prior to the start of the study, animals were allocated randomly to treatment groups as they came to hand (Table 3) and individually tail-marked with a number using an indelible pen.

2.2.9 Experimental design

Numerous attempts, over a period of 21 months, using various study designs were used to validate the EAE relapsing/remitting EAE model in the Biozzi (ABH) mouse. Table 3 summarises the number of animals allocated to the treatments groups.

Part	Treatment	Route	Day of Dosing	Number of animals
I	Chopped Biozzi (ABH) mouse spinal cord in FCA / FIA	Intradermal (base of tail)	1 + 8	8 females
II	Homogenised Biozzi (ABH) mouse spinal cord in FCA / FIA	Intradermal (base of tail)	1 + 8	8 females
III	Homogenised Biozzi (ABH) mouse spinal cord in carbonyl iron	Intraperitoneal	1	6 females
	Bovine MBP in FCA / FIA	Intradermal (base of tail)	1 + 8	6 females
	Murine MBP in FCA / FIA	Intradermal (base of tail)	1 + 8	6 females
	Untreated	-	-	2 females
IV	Homogenised Biozzi (ABH) mouse spinal cord in FCA / FIA	Intradermal (base of tail)	1 + 8	10 females
	Untreated	-	-	5 females
V	Homogenised CD-1 mouse spinal cord in FCA / FIA	Intradermal (base of tail)	1 + 8	5 females
	Homogenised guinea-pig spinal cord in FCA / FIA	Intradermal (base of tail)	1 + 8	5 females
	Untreated	-	-	5 females
VI	Homogenised CD-1 mouse spinal cord in FCA/ FIA	Intradermal (base of tail)	1 + 8	8 males
VII	Freeze dried CD-1 mouse spinal cord in FCA/FIA, plus *mycobacterium butyricum* (heat killed)	Subcutaneous (flank)	1 + 8	8 males
	Untreated	-	-	4 males
VIII	Freeze dried CD-1 mouse spinal cord in FCA / FIA plus *mycobacterium butyricum* (heat killed)	Intradermal (base of tail)	1 + 8	8 males
	Untreated	-	-	2 males

Table 3 Treatment groups for Biozzi (ABH) mouse EAE *in vivo* phase (summary table)

If any of the treatments above had shown a potential to reliably induce relapsing/remitting EAE, a more in depth validation would have taken place and the animals would have been profiled as per the Lewis rats as previously described.

2.2.9.1 Part I - Encephalitogenic agent = finely chopped Biozzi (ABH) mouse spinal cord plus FCA or FIA (10 mg/mL)

Although there are numerous methods for inducing EAE in the Biozzi (ABH) mouse this method, based on published information [Liblau *et a*l. (1997) and Heremans *et al.* (1999)] and modified based on previous experience with other immunological animal models (e.g. collagen type II arthritis in the rat), was selected for this project.

Food and water were available *ad libitum*, except when the animals were removed from their home cage for the study procedures.

A total of eight female Biozzi (ABH) mice (approximately 6 weeks old and 21-25 g on Day 1), housed four per cage was used for this phase of the validation.

On Day 1, eight female Biozzi (ABH) mice received a single 0.1 mL injection of finely chopped Biozzi (ABH) mouse spinal cord in FCA (10 mg/mL) intradermally into the base of the tail. On Day 8, the mice received a booster injection into the base of the tail of 0.1 mL of finely chopped Biozzi (ABH) mouse spinal cord in FIA (10 mg/mL).

The mice were observed daily from Day 1 and scored for signs of EAE as follows:

0 Normal

1 Slight limpness of tail (distal half)

2 Moderate limpness of the tail

3 Substantial limpness of tail (entire tail)

4 Ataxia/hind limb weakness

5 Extreme weakness of hind limbs, but not paralysis (response to toe pinch)

6 Hind limb paralysis (dragging hind limbs behind when moving).

It is possible for any one animal to have more than one of the scores shown above and in such instances the scores are added together. For example, an animal displaying a substantially limp tail and an ataxic gait would have a total score of 7.

The scoring system used was developed on the basis of previous experience with this model.

Bodyweights were recorded twice weekly.

Animals were killed on Day 73.

2.2.9.2 Part II - Encephalitogenic agent = homogenised Biozzi (ABH) mouse spinal cord plus FCA or FIA (10 mg/mL)

Due to a lack of EAE signs in Part I, it was thought that this might have been due to poor preparation of the spinal cord. For this phase of the

validation spinal cord was homogenised, rather than finely chopped, to form a uniform and fine suspension.

A total of eight female Biozzi (ABH) mice (approximately 6 weeks old and 13-24 g on Day 1), housed four per cage was used for this phase of the validation.

Food and water were available at all times, except when the animals were removed from their home cage for study procedures.

On Day 1, eight female Biozzi (ABH) mice received a single 0.1 mL injection of homogenised Biozzi (ABH) mouse spinal cord in FCA (10 mg/mL) intradermally into the base of the tail. On Day 8, the mice received a booster injection into the base of the tail of 0.1 mL of homogenised Biozzi (ABH) mouse spinal cord in FIA (10 mg/mL).

The mice were observed daily from Day 1 and scored for signs of EAE as previously described.

Bodyweights were record twice daily.

Four animals were killed on Day 87 and four on Day 89.

2.2.9.3 Part III - Encephalitogenic agents = homogenised Biozzi (ABH) mouse spinal in carbonyl iron or bovine MBP in FCA / FIA or murine MBP in FCA / FIA.

Due to limited success in Part II of the validation, it was considered necessary to change the EAE induction protocol. Three different methods of induction were examined. Spinal cord homogenised with carbonyl iron dosed intraperitoneally was assessed; this being the method of induction in the rat EAE model which was found to be successful. The use of bovine and murine myelin basic protein have been documented in published literature [Asensio *et al*. (1999)] and as such were also considered worthy of investigation.

A total of 20 female Biozzi (ABH) mice (approximately 6 weeks old and 19-24 g on Day 1), housed up to three per cage was used for this phase of the validation.

Food and water were available at all times, except when the animals were removed from their home cage for study procedures.

On Day 1, female Biozzi (ABH) mice received a single 0.2 mL intraperitoneal injection of homogenised Biozzi (ABH) mouse spinal cord (100 mg/mL) in carbonyl iron (100 mg/mL), bovine MBP (1 mg/mL) in FCA intradermally into the base of the tail, or murine MBP (1 mg/mL) in FCA intradermally into the base of the tail.

On Day 8, the mice dosed with bovine or murine MBP received a booster injection of bovine MBP (1 mg/mL) in FIA or murine MBP (1 mg/mL) in FIA, respectively, intradermally into the base of the tail.

Two mice remained untreated and acted as negative controls.
The mice were observed daily from Day 1 and scored for signs of EAE as previously described.

Bodyweights were record daily from Day 11 to Day 56.

Animals were killed on Day 62.

2.2.9.4 Part IV - Encephalitogenic agent = homogenised Biozzi (ABH) mouse spinal cord plus FCA / FIA (10 mg/mL)

Due to limited success it was considered appropriate to return to the original induction protocol using female mice. However, in this phase of the study animals were weighed daily to see if a cyclic decrease in bodyweight was present, even in the absence of EAE specific signs.

A total of 15 female Biozzi (ABH) mice (approximately 5 weeks old and 19-23 g on Day 1), housed five per cage was used for this phase of the validation.

Food and water were available at all times, except when the animals were removed from their home cage for study procedures.

On Day 1, ten female Biozzi (ABH) mice received a single 0.1 mL injection of homogenised Biozzi (ABH) mouse spinal cord in FCA (10 mg/mL) intradermally into the base of the tail. On Day 8, the mice received a booster injection intradermally into the base of the tail of 0.1 mL of homogenised Biozzi (ABH) mouse spinal cord in FIA (10 mg/mL).

Five mice remained untreated and acted as negative controls.

The mice were observed daily from Day 1 and scored for signs of EAE as previously described.

Bodyweights were record daily.

Three animals were killed on Day 84, one animal on Day 91, two animals on Day 93 and the remainder on Day 109.

From Day 78 onwards, encephalitogenic-treated mice exhibited hair loss around the whisker area.

2.2.9.5 Part V - Encephalitogenic agent = homogenised CD-1 mouse or guinea-pig spinal cord plus FCA / FIA (10 mg/mL)

A possible cause of the lack of anticipated response in the previous validation attempts was thought possibly to be due to the encephalitogenic agent being too similar to ‘self’, i.e. Biozzi (ABH) spinal cord. For the acute EAE study, rats are injected with guinea-pig spinal cord and

successfully develop EAE signs. In order to investigate this further, for this phase of the study CD-1 mouse and guinea-pig spinal cord was used.

A total of 15 female Biozzi (ABH) mice (approximately 6 weeks old and 20-25 g on Day 1), housed five per cage was used for this phase of the validation.

Food and water were available at all times, except when the animals were removed from their home cage for study procedures.

On Day 1, female Biozzi (ABH) mice received a single 0.1 mL injection of homogenised CD-1 mouse or guinea-pig spinal cord in FCA (10 mg/mL) intradermally into the base of the tail. On Day 8, the mice received a booster injection intradermally into the base of the tail of 0.1 mL of homogenised CD-1 mouse or guinea-pig spinal cord in FIA (10 mg/mL).

Five mice remained untreated and acted as controls.

The mice were observed daily from Day 1 and scored for signs of EAE as previously described.

Bodyweights were record daily.

Animals were killed on Day 51.

2.2.9.6 Part VI - Encephalitogenic agent = homogenised CD-1 mouse spinal cord plus FCA / FIA (10 mg/mL)

Following advice from a contact at St Bartholomew's Hospital Medical College, London, validation was attempted using male Biozzi (ABH) mice. Although female mice are in general more susceptible, success of inducing EAE in females can be variable due to fluctuating hormone levels. The mice were housed individually to prevent in-house fighting, thus minimising the potential to increase stress hormone levels such as corticosteroids, which have the affect of inhibiting the required autoimmune response. The animals used were considerably older than previously tested and were considered to be an optimum age for EAE induction.

A total of eight male Biozzi (ABH) mice (approximately 10 weeks old and 29-33 g on Day 1) was used for this phase of the validation.

Food and water were available at all times, except when the animals were removed from their home cage for study procedures.

On Day 1, male Biozzi (ABH) mice received a single 0.1 mL injection of homogenised CD-1 mouse spinal cord in FCA (10 mg/mL) intradermally into the base of the tail. On Day 8, the mice received a booster injection intradermally into the base of the tail of 0.1 mL of CD-1 mouse spinal cord in FIA (10 mg/mL).

The mice were observed daily from Day 1 and scored for signs of EAE as previously described.

Bodyweights were record daily.

Animals were killed on Day 23.

2.2.9.7 Part VII - Encephalitogenic agent = Freeze dried CD-1 mouse spinal cord plus FCA / FIA (10 mg/mL), plus 3 mg/mL Mycobacterium butyricum (heat killed)

Due to a failure of EAE induction, further advice was received which indicated that freeze dried spinal cord was preferable to homogenised material. Also Freund's adjuvant should be supplemented with *Mycobacterium*. Immunisation by subcutaneous administration into each flank was suggested [Liblau *et a*l. (1997) and Heremans *et al.* (1999)].

A total of 12 male Biozzi (ABH) mice (approximately 12 weeks old and 27-35 g on Day 1), housed individually was used for this phase of the validation.

Food and water were available at all times, except when the animals were removed from their home cage for study procedures.

On Day 1, male Biozzi (ABH) mice received a single 0.1 mL injection of freeze dried CD-1 mouse spinal cord in FCA (10 mg/mL), plus 3 mg/mL

mycobacterium butyricum (heat killed) subcutaneously into both the shaved flanks.

On Day 8, the mice received a booster injection subcutaneously into both the shaved flanks of 0.1 mL of freeze dried CD-1 mouse spinal cord in FIA (10 mg/mL), plus 3 mg/mL *Mycobacterium butyricum* (heat killed).

Four mice remained untreated and acted as negative controls.

The mice were observed daily from Day 1 and scored for signs of EAE as previously described.

Bodyweights were record daily.

Animals were killed on Day 27.

2.2.9.8 Part VIII - Encephalitogenic agent = freeze dried CD-1 mouse spinal cord plus FCA / FIA (10 mg/mL) 3 mg/mL Mycobacterium butyricum (heat killed)

A lack of EAE signs in mice in Part VII led to a final attempt at inducing EAE in the Biozzi (ABH) mouse. For this phase, intradermal injection into the base of the tail was used as before, but this time *Mycobacterium* was added to the Freund's adjuvant at a concentration of 3 mg/mL.

A total of 10 male Biozzi (ABH) mice (approximately 10 weeks old and 30-37 g on Day 1), housed individually was used for this phase of the validation.

Food and water were available at all times, except when the animals were removed from their home cage for study procedures.

On Day 1, eight male Biozzi (ABH) mice received a single 0.1 mL injection of freeze dried CD-1 mouse spinal cord in FCA (10 mg/mL) plus 3 mg/mL *Mycobacterium butyricum* (heat killed) intradermally into the base of the tail. On Day 8, the mice received a booster injection into the base of the tail of 0.1 mL of freeze-dried CD-1 mouse spinal cord in FIA (10 mg/mL) plus 3 mg/mL *Mycobacterium butyricum* (heat killed).

Two mice remained untreated and acted as controls.

The mice were observed daily from Day 1 and scored for signs of EAE as previously described.

Bodyweights were record daily.

Two animals were killed on Day 18 and the remainder on Day 25.

2.2.10 Source of spinal cord and brains from Biozzi (ABH) mice

Due to a lack of success in validating the EAE model in the Biozzi (ABH) mouse. The spinal cords and brains of two Biozzi (ABH) mice which

exhibited peak EAE signs during a second EAE episode (relapse) were donated by Chris Bolton at John Vane Science Centre, William Harvey Research Institute, St Bartholomew's Hospital Medical College, London. Spinal cord and brains were also supplied from control (untreated) Biozzi (ABH) mice.

2.3 Method Validation of Anti-ss and –dsDNA Antibodies in Rat Sera and CSF using an ELISA Method

2.3.1 Materials

Plasmid dsDNA, 1 mg/mL (lot 5) and plasmid ssDNA, 0.5 mg/mL (lot 6) were supplied by Bayou Biolabs, USA. Plasmid DNA was produced from E coli.

Linearisation of dsDNA: Restriction endonuclease Bam H1 (batch 41K1292) and Solubilisation/Denaturation (SD) buffer (batch 107H0592) were supplied by Sigma, UK.

Electrophoresis: Agarose (batch 3060113, supplied by Life Technologies), Tris-borate-EDTA (batch 3066660, Invitrogen–Life Technologies), ethidium bromide (batch 1113071, Invitrogen–Life Technologies), λDNA/HIND III fragments (batch D-9780, Sigma, UK), DNase-, RNase-free 0.2 μm filtered water (batch 91K2328, Sigma, UK), gel loading solution (batch 02411, Biotech) and non-sterile reverse osmosis high purity (ROHP) water for gel preparation (batch 1284, Covance, UK).

Photobiotinylation of dsDNA: Photobiotin (batch 60K5004), butanol (batch U01195), ethanol (batch 111K3657) and Tris EDTA (batch 21K8933) were supplied by Sigma, UK.

ELISA assay: Streptavidin (batch 118H8660), gelatin (batch 50K0124), phosphate buffered saline (batch 71K8201), Tween 20 (batch 81K8201), o-phenylenediamine (batch 11K5312), citrate phosphate buffer (batch 99H8203), H_2SO_4 (batch 071K3251) and Tris EDTA (batch 21K8933) were supplied by Sigma, UK. HCl (batch K28735352103) was supplied by BDH Laboratory supplies, UK. Horseradish peroxidase conjugate IgG (Lot 128 (902)), IgA (Lot 055 (602)) and IgM (Lot 080 (501)) were supplied by Dako, Denmark. Plasmid ds and ssDNA was obtained from Bayou Biolabs, USA. Anti-dsDNA antibody (batch 454920) was supplied by The Binding Site, UK. Anti-ssDNA antibody (batch 1678) was supplied by Immunovision, Inc, USA.

2.3.2 Linearisation of plasmid dsDNA

Fresh uncut plasmid dsDNA preparation can be present in a number of forms; nicked, closed circular, linear, covalently bonded, supercoiled or circular single-stranded. Nicked or opened DNA occurs when there is a break in one or more strands which causes the DNA to unwind. Closed circular DNA is formed by two strands of the double helix linking to form a circle. Supercoiled DNA occurs when the double helix becomes twisted around itself in a high-energy state. Covalently bonded DNA is when DNA becomes strongly linked with RNA or protein. Circular single-stranded is when a single strand links together to form a circle and linear DNA is a

straight piece of DNA. When uncut plasmid dsDNA is run on a gel in electrophoresis, multiple bands relating to these different DNA forms can often be seen, as shown in figure 1.

A linear form of plasmid was needed for photobiotinylation, so the plasmid dsDNA was cut to a linear form with the restriction endonuclease Bam H1. Restriction endonuclease Bam H1 (from *Bacillus amyloli)* works by recognising short sections (6 base pairs) of DNA and cleaving them at the target site. The resulting preparation formed a single band on the gel when run following digestion as shown in figure 2 (original photographs unavailable).

Linear DNA was prepared by dividing 200 μL of dsDNA (1 mg/mL) equally into four tubes. To each tube, 20 μL (200 units) of restriction endonuclease Bam H1, 32.5 μL water and 97.5 μl of SD buffer was added. Although 1 unit of Bam H1 cleaves 1 μg of DNA, a higher concentration was recommended. Care was taken to ensure that the amount of enzyme added was no more that 10% of the total volume, as exceeding this amount could inhibit the reaction due to the glycerol present in the storage buffer. The digestion process was performed by placing the tubes containing the mixture in an incubation cabinet at 37°C overnight. The following day the reaction was inactivated by placing the tubes in a water-bath (Techne) at 65°C for 20 minutes. The mixture was stored frozen at -70°C prior to electrophoresis.

2.3.3 Electrophoresis - linearisation check

Linearisation of plasmid dsDNA was examined using agarose gel electrophoresis. The gel consisted of 0.8% agarose in 0.5 x Tris-borate-EDTA pH 8.0 and was heated until it was dissolved. The agarose was poured into a gel tray with an appropriate comb in the presence of 0.5 μg/mL of ethidium bromide and allowed to set.

10 μL of DNA sample was mixed with 2 μL gel loading buffer which consisted of 0.25% bromophenol, 0.25% xylene cyanol and 15% Ficoll type 400 in water. 6 μL of mixture were added to a slot of the gel.

λDNA/HIND III fragments were used as standard molecular weight markers and 0.1 μg/mL of the fragments were mixed with gel loading buffer and loaded onto the gel.

The electrophoresis was carried out until the bromophenol blue had migrated to the opposite end of the gel. A constant voltage of 100 volts was applied for a period of 1 hour using a wide-mini sub cell gel tank (Bio-Rad).

The gel was viewed was under short-wavelength ultraviolet irradiation and a photograph was taken using a Polaroid CU-5 camera (actual photographs inadvertently misplaced).

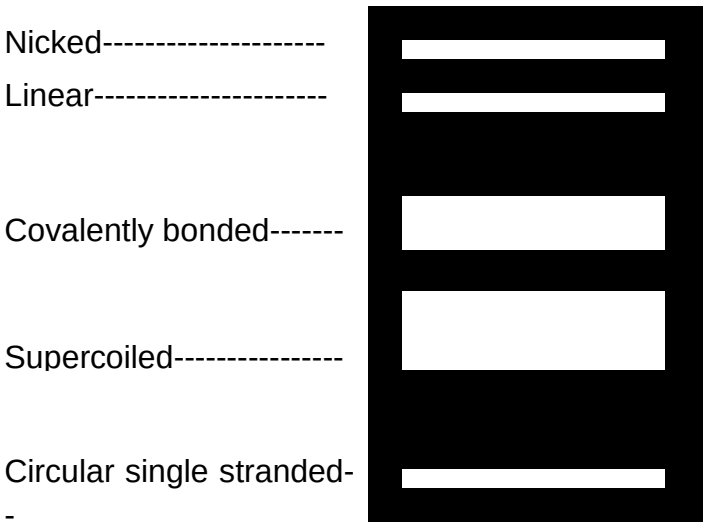

Figure 1 A diagram showing the appearance of uncut plasmid DNA. Fresh uncut plasmid dsDNA preparation can be nicked, circular, linear, covalently closed supercoiled or circular single stranded and when run on agarose gel in electrophoresis, multiple bands can often be seen.

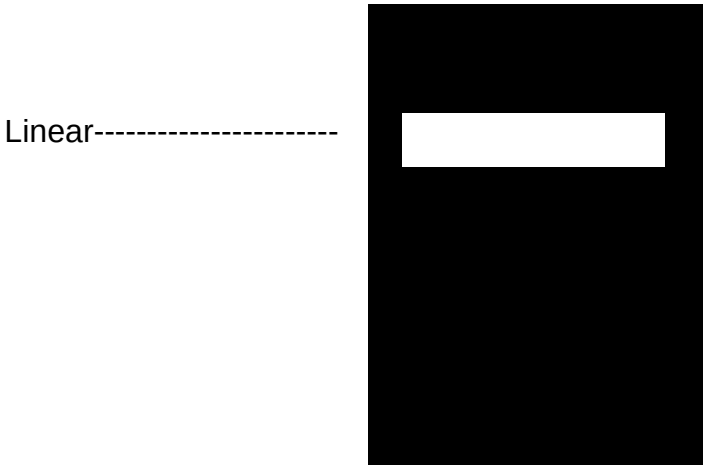

Figure 2 A diagram showing the appearance of cut plasmid DNA. Following linearisation of dsDNA using restriction endonuclease Bam H1, a single band is formed when run on agarose gel.

2.3.4 Photobiotinylation of plasmid DNA

Plasmid ds and ssDNA was previously prepared at a concentration of 0.5 mg/mL. Each was mixed with an equal volume of photobiotin, at a concentration of 1 mg/mL, without exposure to light. The mixture was placed on ice and irradiated with a 250W light source through glass to exclude UVB, at a distance of 10 cm for 20 minutes.

An equal volume of butanol was added to extract the DNA and to remove the unbound photobiotin. This was repeated a further two times. The plasmid DNA was then ethanol precipitated by incubation at 4°C for 1 hour, followed by centrifugation at 12000 g for 30 minutes at 4°C. After rinsing with 70% ethanol and another centrifugation at 12000 g for 10 minutes at 4°C, the plasmid DNA was re-suspended in Tris-EDTA, pH 8.0 and its concentration determined by measuring the absorbance at 260 nm.

The concentration of plasmid DNA was measured by reading the absorbance at 260 nm using a spectrophotometer (Pharmacia). 50 µg/mL of dsDNA is known to have an absorbance of 1 at 260 nm wavelength and 40 µg/mL of ssDNA is known to have an absorbance of 1 at the same wavelength.

Photobiotinylated DNA was stored frozen at -70°C prior to use in the ELISA.

2.3.5 ELISA validation

The ELISA used was based on the method detailed by Emlen *et al.* (1990).

For the validation of the ELISA, the procedure detailed above was followed. Response curves were produced using various dilutions (in duplicate) of anti-dsDNA antibodies and anti-ssDNA antibodies as follows:

dsDNA ELISA reciprocal dilutions: 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048, 4096, 8192, 16384 and 32768.

ssDNA ELISA reciprocal dilutions: 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, 131072.

The response curves obtained in the ELISA validation (figure 3) were as expected with the anti-dsDNA antibodies exhibiting a slightly higher affinity for the antigen (dsDNA), compared to the affinity demonstrated by the anti-ssDNA antibodies for their associated antigen (ssDNA). This effect was particularly apparent at reciprocal dilutions of 100 – 10,000.

In general, the data showed little variability, the exception being for anti-ssDNA at a reciprocal dilution of 8. The individual OD values of the duplicates were 1.090 and 1.510, with the 1.090 OD value appearing to be erroneous. This variation may have been due to an insufficient sample

being added to the well during pipetting or perhaps poor coating of this particular well with antigen, thus providing limiting binding sites.

Due to limited volumes available, all samples were analysed in duplicate rather than triplicate.

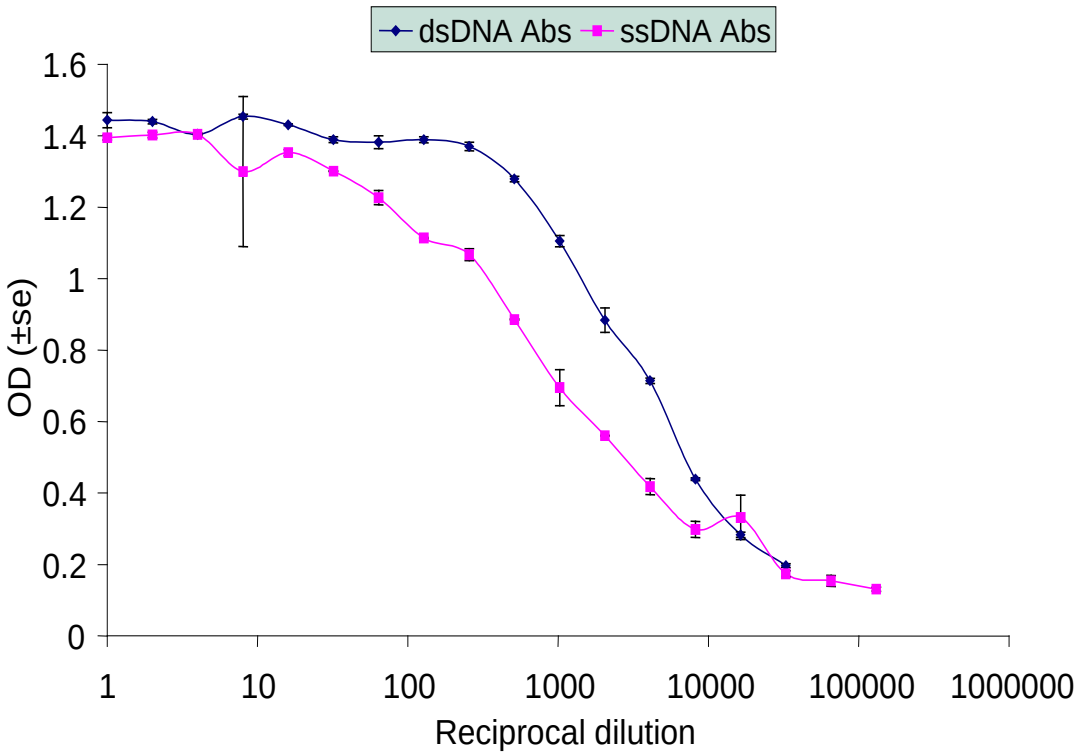

Figure 3 Anti-dsDNA and anti-ssDNA antibody ELISA validation curves. Response curves were produced using various dilutions of anti-dsDNA antibodies (reciprocal dilutions: 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048, 4096, 8192, 16384 and 32768) and anti-ssDNA antibodies (reciprocal dilutions: 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, 131072.). The response curves indicate that anti-dsDNA antibody has a higher affinity for the antigen, compared to the anti-ssDNA antibody. Each dilution sample was analysed in duplicate.

In addition, the potential of cross-reaction between ss and dsDNA ELISA plates were examined. This was done by adding reciprocal dilutions (1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, 131072) of anti-dsDNA antibodies to ssDNA coated plates and reciprocal dilutions (1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, 131072) of anti-ssDNA antibodies to dsDNA coated plates.

Cross reactivity investigation showed there to be minimal cross-reaction when dsDNA antibodies were added to ssDNA coated plates (figure 4). However, when ssDNA antibodies were examined for cross-reactivity, a similar curve was obtained to that when dsDNA antibodies are added at the same concentrations to a plate coated with dsDNA.

In general, the data showed little variability, the exception being for ssDNA versus dsDNA antibody at a reciprocal dilution of 64, and dsDNA versus ssDNA antibody at reciprocal dilutions of 128, 2048 and 4096. The individual OD values of the duplicates for ssDNA versus dsDNA antibody at a reciprocal dilution of 64 were 0.430 and 0.943, with both values being markedly higher than expected. This variation between the duplicates may have been due to inaccurate pipetting.

The individual OD values of the duplicate samples for ssDNA versus dsDNA antibody at a reciprocal dilution of 128 were 0.901 and 1.140, and at reciprocal dilutions of 2048 and 4096 were comparable to each other

with OD values of 0.450, 0.610 and 0.609, 0.451, respectively. Pipetting technique is a probable cause of the variability in all cases.

In addition to the variability seen for a minority of duplicates, the response curve for ssDNA versus dsDNA antibody had two outliers, one at a reciprocal dilution of 64 and the other at 16384. The mean of the duplicates at each dilution and the OD values of each individual sample was much higher that anticipated. These results suggest that the error did not occur during the well preparation or during pipetting, but more likely occurred at the dilution stage.

All samples were analysed in duplicate.

The results suggest that if both anti-ss and –dsDNA antibodies were present in a sample, it would not be possible to detect anti-ssDNA antibody alone using this method. However, using an anti-dsDNA plate the presence of anti-dsDNA antibodies could be reliably detected using this method.

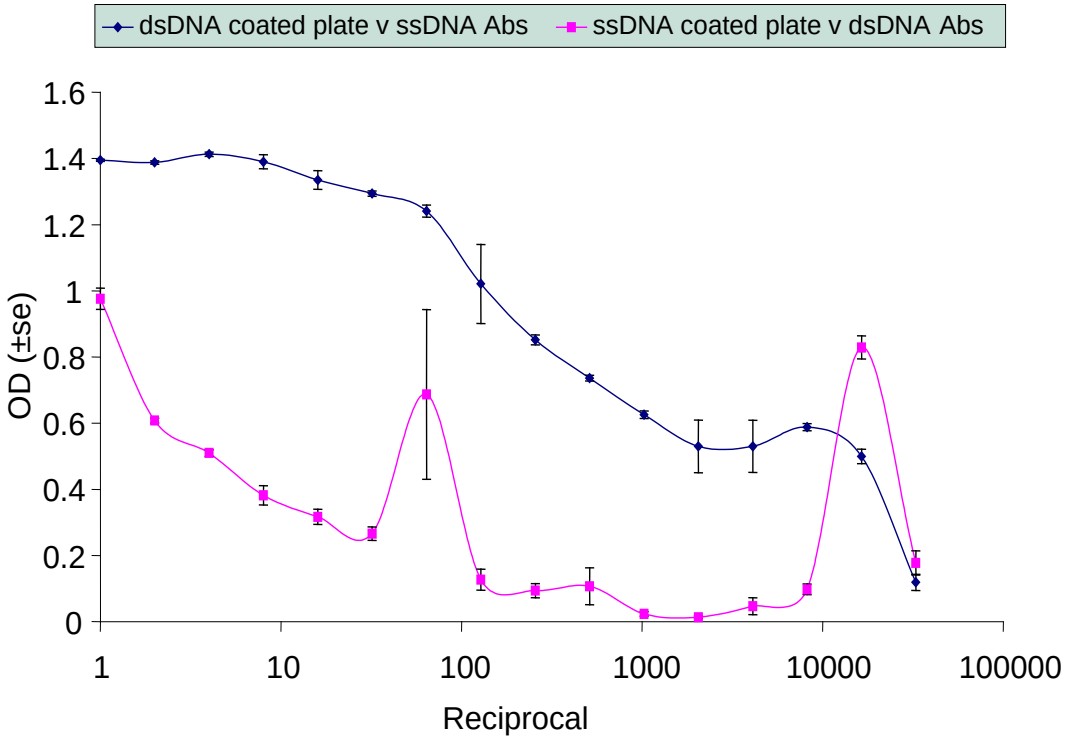

Figure 4 Anti-dsDNA and anti-ssDNA antibody cross-reactivity ELISA response curves. Response curves were produced by adding reciprocal dilutions (1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, 131072) of anti-dsDNA antibodies to ssDNA coated plates and reciprocal dilutions (1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, 131072) of anti-ssDNA antibodies to dsDNA coated plates. Minimal cross-reaction occurred when dsDNA antibodies were added to ssDNA coated plates, whereas a high level of cross-reactivity occurred when ssDNA antibodies were added to dsDNA coated plates. Each dilution sample was analysed in duplicate.

2.3.6 Detection of anti-DNA antibody by ELISA using photobiotinylated DNA as the antigen

Streptavidin (to which biotinylated DNA forms non-covalent bonds, thus attaching the DNA to a streptavidin coated surface) was diluted with distilled water to a concentration of 1 μg/mL. 150 μL/well of streptavidin was added to Maxisorp 96-well plates (Nunc) which were then incubated at 4°C overnight. After four washes with PBS/0.05% Tween 20, 200 μL/well of 0.2% gelatin in PBS was added to the plates as a blocking reagent, this was to prevent any other reagents from attaching to the surface not occupied by streptavidin, which could lead to erroneous readings. After four washes with PBS/0.05% Tween 20, 150 μL/well of a 1 μg/mL solution of photobiotinylated DNA was added to each well of a Maxisorp plate and incubated at 4°C overnight. After another four washes with PBS/Tween 20, 50 μL of supernatant (CSF or sera) was added to the wells and incubated at room temperature for 2 hours. After four washes with PBS/Tween 20, 50 μL/well of horseradish peroxide conjugated human IgG, A, M diluted to 1:1000 with blocking buffer was added and the plates were incubated for 1 hour at room temperature. After four washes, 50 μL/well of 1 mg/mL OPD in 0.5M of citrate phosphate buffer (pH 5.0) in the presence of 0.003% H_20_2 (prepared immediately before use) was added to the plates and incubated for 15 minutes at room temperature in the dark. 25 μL/well of 1M HCl was added to stop the reaction and the plates were read at 492 nm wavelength.

Sera and CSF samples were initially examined at 1:50 dilution in duplicate. Undiluted sera and CSF, where possible were subsequently assayed. Each run included duplicate blank wells and seven dilutions of the appropriate reference (anti-dsDNA or anti-ssDNA antibodies). For ssDNA plates the following reciprocal dilutions were used for the response curve; 10, 100, 500, 1000, 4000, 8000 and 40000. For dsDNA plates the following reciprocal dilutions were used: 100, 500, 1000, 4000, 8000, 15000 and 50000. Three dilutions of the reference standards were positioned in duplicate at the beginning and at the end of each run. For the ssDNA plates these were 250, 2000 and 10000 reciprocal dilutions and 750, 5000 and 10000 reciprocal dilutions for the dsDNA plates.

2.4 Method Validation of Immuno-histopathology Techniques in Rat CNS Tissue

2.4.1 Materials

3,3-diaminobenzidine (DAB, batch 32K1582 and 71K1383, Sigma, UK), Dulbecco's phosphate buffer saline (batch 80K83031, Sigma, UK), hydrogen peroxide (batch 052K3250, Sigma, UK), methanol (batch 3F26MA, 2J30HA, 2K01HA and 2G11SA, Rathburn Chemicals Ltd., UK), acetone (batch 2E29EB, 2J19MB and 2G09HB, Rathburn Chemicals Ltd., UK), rabbit anti-mouse Igs-biotin, code E0464 (batch 079(201) and 059/601 Dako), StreptABComplex/HRP (batch 100(101), Dako), mouse anti-rat B cells (batch MCA1432 and 041102, Serotec), normal rabbit serum (batch M1128 and N0404, Vector Laboratories Inc. USA), donkey anti-sheep Igs (batch 050385, Binding Site, UK), sheep anti-rat complement C3 (batch 046536, Binding Site, UK), normal donkey serum (batch 080202, Serotec), avidin/biotin (batch N0503, Vector Laboratories Inc, USA), sheep anti-rat IgG:HRP (batch AAR10P, Serotec), normal sheep serum (batch 250401, Serotec), mouse anti-rat IgM:HRP (batch MCA189P and 220502, Serotec), mouse serum (batch 011(102), 041(101) and 062(101), Dako, Denmark). 3-aminopropyl triethoxysilane (APES: batch 91K1271, Sigma), DPX (batch 140398, Surgipath Europe Ltd, UK), 4',6-diamidino-2-phenylindole (DAPI: batch 12K4067, Sigma), Dako fluorescent mounting medium (batch 06260, Dako).

Copper sulphate, Scott's tap water, haematoxylin and eosin were formulated in bulk by the Histology Department at Covance. Copper sulphate (0.5% w/v) solution was prepared by dissolving copper sulphate in 0.9% (w/v) sodium chloride. Scott's tap water consisted of 2% (w/v) magnesium sulphate $7H_2O$ and 0.2% (w/v) sodium hydrogen carbonate in tap water. Haematoxylin (0.3% w/v) was formulated in 25% (v/v) ethanediol (ethan-1,2-diol), 2% (v/v) glacial acetic acid, 0.02% (w/v) sodium iodate and 3.5% (w/v) aluminium sulphate in deionised water. Eosin (1% w/v) was formulated in 95% industrial methylated spirits (IMS).

2.4.2 Sectioning

5 micron sections of spinal cord from each animal were sectioned at 50 micron intervals using a cryostat, sections were placed on aminopropyltriethoxysilane (APES) coated slides. Slides were stored frozen (nominally -70ºC) prior to staining.

APES solution was formulated at a concentration of 2% v/v in acetone. Clean slides were immersed in the APES solution for 5 minutes and then allowed to drain, but not dry. The slides were washed in running tap water and allowed to air dry overnight. APES is a section adhesive and is used when there is an increased risk of the tissue section lifting from the slide during drying or staining, CNS tissue being prone to this. APES is a polyvalent cationic polymer which gives the coated slide a weak positive charge. Section binding occurs by ionic interaction between the charged slide and the negatively charged functional groups (eg carboxyl, hydroxyl) within the tissue section.

2.4.3 Determination of IgM

Rat spinal cord slides were removed from the freezer and allowed to air dry for at least 2 hours. Slides were then placed in approximately 200 mL of acetone for 10 minutes to fix the sections and left overnight at room temperature to air dry.

The following day the slides were washed with Dulbecco's buffer for 10 minutes. Slides were blocked for endogenous enzyme, using 750 µL of hydrogen peroxide in 200 mL methanol (0.1125%) for 30 minutes. Slides were then washed with running tap water for 10 minutes and returned to Dulbecco's buffer. Slides were placed in slide holders and washed with Dulbecco's buffer for 10 minutes. Slides were serum blocked by adding at least 100 µL of 20% normal mouse serum in Dulbecco's buffer for 30 minutes. The slides were then washed with Dulbecco's buffer for 10 minutes. At least 200 µL of the primary antibody, mouse anti-rat IgM:HRP in Dulbecco's buffer at a dilution of 1:100, was added to each slide, with the exception of the spleen and spinal cord negative controls to which at least 200 µL of Dulbecco's buffer was added. The slides were placed in the fridge (nominally 2 to 8º) overnight.

The following day the slides were removed from the fridge and washed with Dulbecco's buffer for 10 minutes. The slides were removed from the slide holders and placed in Dulbecco's buffer. Slides were placed in the chromogen DAB for 10 minutes. DAB was prepared immediately prior to use by adding 0.5 g of DAB to 1 L of buffer containing 1 mL of 30%

hydrogen peroxide solution. Once removed from DAB the slides were washed in running tap water for 5 minutes. The slides were then placed in the chromogen enhancer, copper sulphate for 5 minutes, following which the slides were washed in running tap water for 5 minutes. The slides were counter-stained with haematoxylin for 1 minute, rinsed briefly in tap water and placed in Scott's tap water for 1 minute and finally briefly rinsed in tap water.

2.4.4 Determination of IgG

Rat spinal cord slides were removed from the freezer and allowed to air dry for at least 2 hours. Slides were then placed in approximately 200 mL of acetone for 10 minutes to fix the sections and left overnight at room temperature to air dry.

The following day the slides were washed with Dulbecco's buffer for 10 minutes. Slides were blocked for endogenous enzyme, using 750 µL of hydrogen peroxide in 200 mL methanol (0.1125%) for 30 minutes. Slides were then washed with running tap water for 10 minutes and returned to Dulbecco's buffer. Slides were placed in slide holders and washed with Dulbecco's buffer for 10 minutes. Slides were serum blocked by adding at least 100 µL of 20% normal sheep serum in Dulbecco's buffer for 30 minutes. The slides were then washed with Dulbecco's buffer for 10 minutes. At least 200 µL of the primary antibody, sheep anti-rat IgG:HRP in Dulbecco's buffer at a dilution of 1:800 was added to each slide, with the exception of the spleen and spinal cord negative controls to

which at least 200 μL of Dulbecco's buffer was added. The slides were placed in the fridge (nominally 2 to 8º) overnight.

The following day the slides were removed from the fridge and washed with Dulbecco's buffer for 10 minutes. The slides were removed from the slide holders and placed in Dulbecco's buffer. Slides were placed in the chromogen DAB for 10 minutes. DAB was prepared immediately prior to use. Once removed from DAB the slides were washed in running tap water for 5 minutes. The slides were then placed in the chromogen enhancer, copper sulphate for 5 minutes, following which the slides were washed in running tap water for 5 minutes. The slides were counter-stained with haematoxylin for 1 minute, rinsed briefly in tap water and placed in Scott's tap water for 1 minute and finally briefly rinsed in tap water.

2.4.5 Determination of C3

Rat spinal cord slides were removed from the freezer and allowed to air dry for at least 2 hours. Slides were then placed in approximately 200 mL of acetone for 10 minutes to fix the sections and left overnight at room temperature to air dry.

The following day the slides were washed with Dulbecco's buffer for 10 minutes. Slides were blocked for endogenous enzyme, using 750 μL of hydrogen peroxide in 200 mL methanol (0.1125%) for 30 minutes. Slides were then washed with running tap water for 10 minutes and returned to Dulbecco's buffer. Slides were placed in slide holders and washed with

Dulbecco's buffer for 10 minutes. Slides were serum blocked by adding at least 100 µL of 20% normal donkey serum in Dulbecco's buffer for 30 minutes. The slides were then washed with Dulbecco's buffer for 10 minutes. Slides were blocked with avidin for 15 minutes, washed with Dulbecco's buffer for 10 minutes and then blocked with biotin by incubating the slides for 15 minutes. Slides were washed with Dulbecco's buffer. At least 200 µL of the primary antibody, sheep anti-rat complement C3 in Dulbecco's buffer at a dilution of 1:1000, was added to each slide, with the exception of the liver and spinal cord negative controls to which at least 200 µL of Dulbecco's buffer was added. The slides were placed in the fridge (nominally 2 to 8º) overnight.

The following day the slides were removed from the fridge and washed with Dulbecco's buffer for 10 minutes. Slides were incubated for 60 minutes with at least 100 µL of the secondary antibody, 1:200 dilution of donkey anti-sheep Igs. The slides were washed with Dulbecco's buffer for 10 minutes. Binding was visualised using at least 100 µL of streptavidin-biotin for 45 minutes. Streptavidin-biotin was prepared in Dulbecco's buffer at least 30 minutes prior to use and was mixed thoroughly. Streptavidin-biotin kit Instructions were followed to ensure that there was an excess of biotin present in order to fully saturate the streptavidin so to prevent non-specific binding. The slides were washed with Dulbecco's buffer for 10 minutes. The slides were removed from the slide holders and placed in Dulbecco's buffer. Slides were placed in the chromogen DAB for 10 minutes. DAB was prepared immediately prior to

use. Once removed from DAB the slides were washed in running tap water for 5 minutes. The slides were then placed in the chromogen enhancer, copper sulphate for 5 minutes, following which the slides were washed in running tap water for 5 minutes. The slides were counter-stained with haematoxylin for 1 minute, rinsed briefly in tap water and placed in Scott's tap water for 1 minute and finally briefly rinsed in tap water.

2.4.6 Determination of B cells

Rat spinal cord slides were removed from the freezer and allowed to air dry for at least 2 hours. Slides were then placed in approximately 200 mL of acetone for 10 minutes to fix the sections and left overnight at room temperature to air dry.

The following day the slides were washed with Dulbecco's buffer for 10 minutes. Slides were blocked for endogenous enzyme, using 750 µL of hydrogen peroxide in 200 mL methanol (0.1125%) for 30 minutes. Slides were then washed with running tap water for 10 minutes and returned to Dulbecco's buffer. Slides were placed in slide holders and washed with Dulbecco's buffer for 10 minutes. Slides were serum blocked by adding at least 100 µL of 20% normal rabbit serum in Dulbecco's buffer for 30 minutes. The slides were then washed with Dulbecco's buffer for 10 minutes. Slides were blocked with avidin for 15 minutes, washed with Dulbecco's buffer for 10 minutes and then blocked with biotin by incubating the slides for 15 minutes. Slides were washed with Dulbecco's buffer. At least 200 µL of the primary antibody, mouse anti-rat B cells in

Dulbecco's buffer at a dilution of 1:80, was added to each slide, with the exception of the liver and spleen cord negative controls to which at least 200 μL of Dulbecco's buffer was added. The slides were placed in the fridge (nominally 2 to 8º) overnight.

The following day the slides were removed from the fridge and washed with Dulbecco's buffer for 10 minutes. Slides were incubated for 60 minutes with at least 100 μL of the secondary antibody, 1:500 dilution of rabbit anti-mouse Igs. The slides were washed with Dulbecco's buffer for 10 minutes. Binding was visualised using at least 100 μL of streptavidin-biotin for 45 minutes. Streptavidin-biotin was prepared at least 30 minutes prior to use and was mixed thoroughly. The slides were washed with Dulbecco's buffer for 10 minutes. The slides were removed from the slide holders and placed in Dulbecco's buffer. Slides were placed in the chromogen DAB for 10 minutes. DAB was prepared immediately prior to use. Once removed from DAB the slides were washed in running tap water for 5 minutes. The slides were then placed in the chromogen enhancer, copper sulphate for 5 minutes, following which the slides were washed in running tap water for 5 minutes. The slides were counter-stained with haematoxylin for 1 minute, rinsed briefly in tap water and placed in Scott's tap water for 1 minute and finally briefly rinsed in tap water.

2.4.7 Final preparation of IgM, IgG, C3 and B cell slides

Slides were placed in five changes of IMS, followed by three changes of xylene. Cover-slips were mounted using DPX. Slides were allowed to dry prior to viewing.

2.4.8 Validation of immuno-staining techniques

Each of the above was validated using various dilutions of the primary antibody.

IgG: various dilutions (1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800) of sheep anti-rat IgG:HRP were used on sections of rat spleen. Spleen was used as this tissue has a high IgG content. Dilutions of 1:400 and 1:12800 were added to spinal cord sections from an untreated rat. In addition, a section of spleen and spinal cord were included as negative controls, i.e. Dulbecco's buffer was added in place of the primary antibody. On the basis of the results obtained a dilution of 1:800 was used for subsequent staining (figure 5).

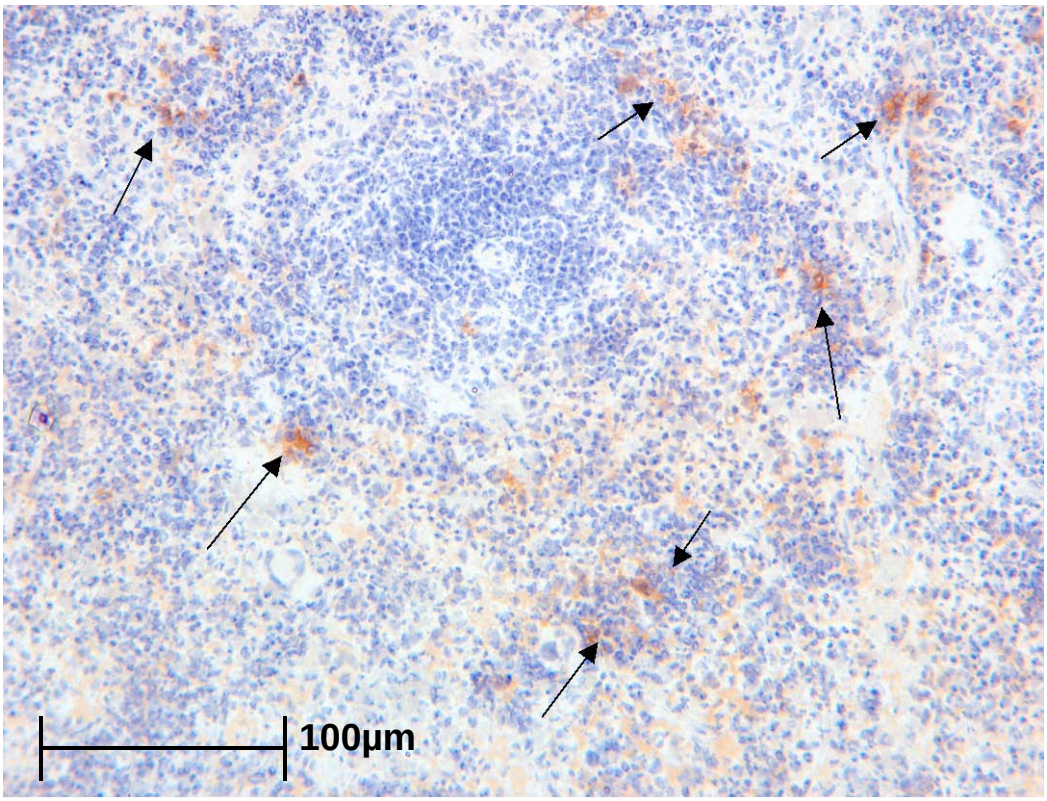

Figure 5 IgG staining (1:800 dilution) using sheep anti-rat IgG:HRP and counter stained with haematoxylin, under light microscopy (x 20 objective). The arrows point to areas of positive IgG staining on a spleen section taken from an untreated rat.

IgM: various dilutions (1:12.5, 1:25, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800) of mouse anti-rat IgM:HRP were used on sections of rat spleen. Spleen was used as this tissue has a high IgM content. Dilutions of 1:200, 1:400 and 1:12800 were added to spinal cord sections from an untreated animal. In addition, a section of spleen and spinal cord were included as negative controls, i.e. Dulbecco's buffer was added in place of the primary antibody. On the basis of the results obtained a dilution of 1:100 was used for subsequent staining (figure 6).

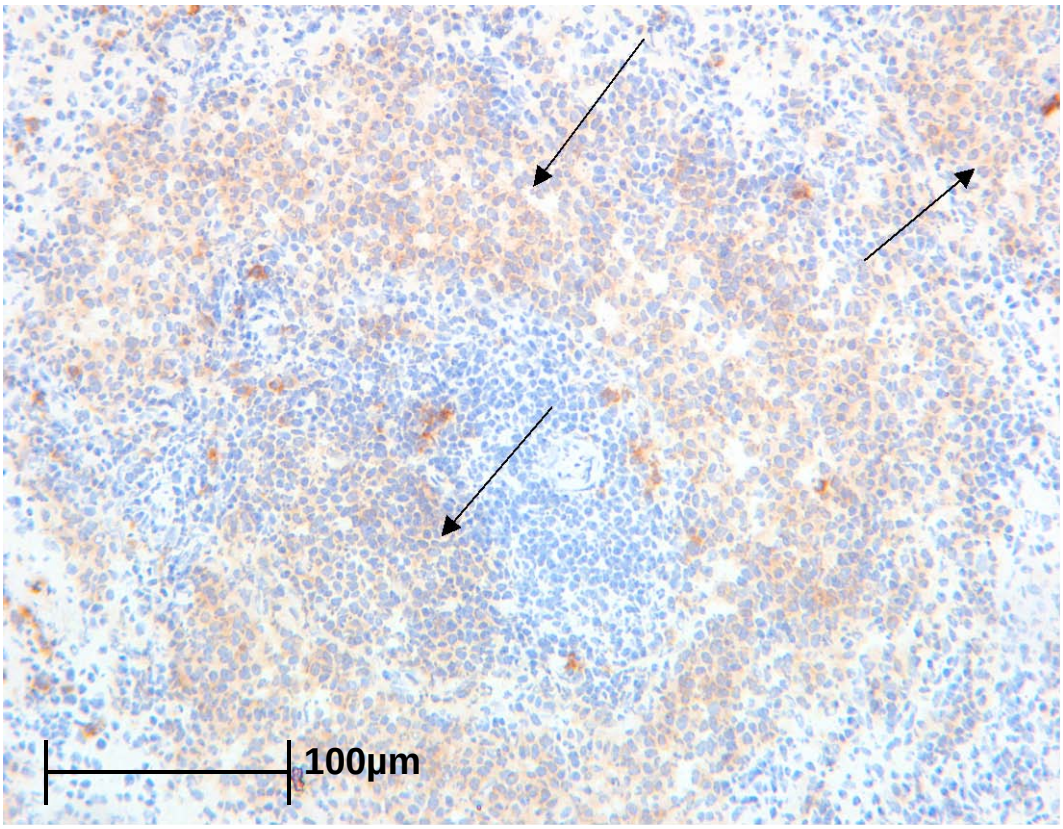

Figure 6 IgM staining (1:100 dilution) using mouse anti-rat IgM:HRP and counter stained with haematoxylin, under light microscopy (x 20 objective). The arrows point to area of positive IgM staining on a spleen section taken from an untreated rat.

B cells, various dilutions (1:10, 1:20, 1:40, 1:80, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200) of mouse anti-rat B cells were used on sections of rat liver. Liver was used as this has a high B cell content. Dilutions of 1:10, 1:80, 1:100 and 1:3200 were added to spinal cord sections from an untreated animal. In addition a section of liver and spinal cord were included as negative controls, i.e. Dulbecco's buffer was added in place of the primary antibody. On the basis of the results obtained a dilution of 1:80 was used for subsequent staining (figure 7).

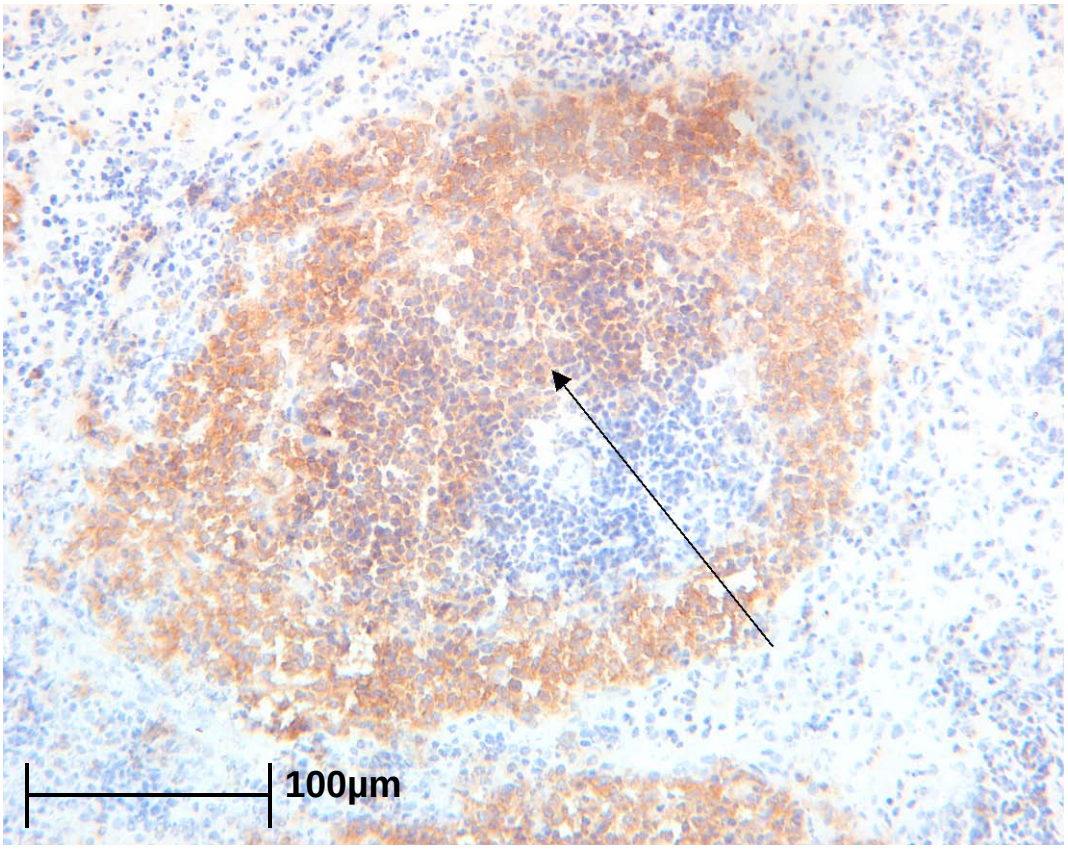

Figure 7 B cell (1:80) staining using mouse anti-rat B cells and counter stained with haematoxylin, under light microscopy (x 20 objective). The arrow points to an area of positive B cell staining on a liver section taken from an untreated rat.

C3: various dilutions (1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800) of sheep anti-rat complement C3 were used on sections of rat spleen. Spleen was used as this tissue has a high C3 content. Dilutions of 1:400 and 1:12800 were added to spinal cord sections from an untreated animal. In addition a section of spleen and spinal cord were included as negative controls, i.e. Dulbecco's buffer was added in place of the primary antibody. On the basis of the results obtained a dilution of 1:1000 was used for subsequent staining (figure 8).

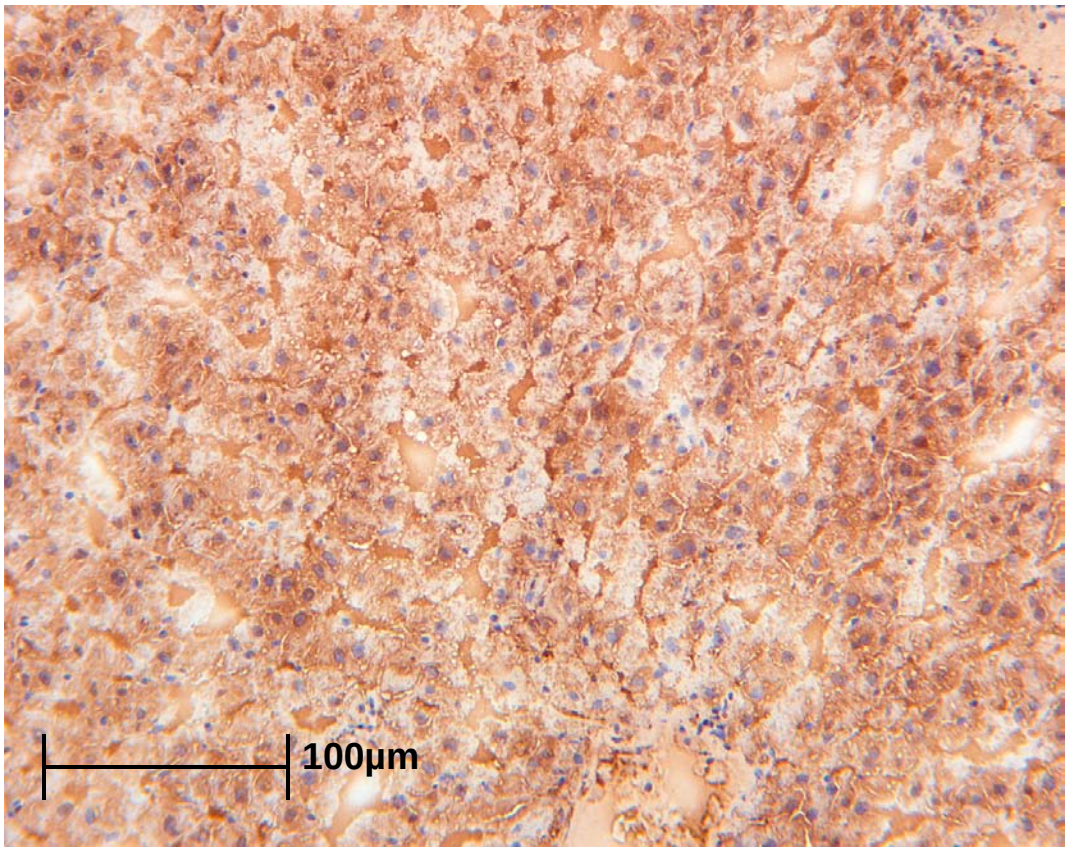

Figure 8 C3 staining (1:1000 dilution) using sheep anti-rat complement C3 and counterstained with haematoxylin, under light microscopy (x 20 objective). The above shows C3 staining on a spleen section taken from an untreated rat.

The dilution of primary antibody selected in each case was one that provided good specific staining with minimal non-specific background staining.

2.4.9 DAPI (4',6-diamidino-2-phenylindole) staining

Due to the limited amount of CSF available from the rat EAE model, it was considered necessary to find an alternative method for the detection of anti-DNA antibodies. DAPI is a fluorescent dye that binds selectively to DNA and forms strongly fluorescent DNA-DAPI complexes with high specificity. Based on this information, the possibility of developing a probe which could act as a specific target for anti-ds or -ssDNA antibodies was investigated. At the time of writing the only references with regards to the use of DAPI in the detection of anti-ss or dsDNA antibodies refer to demonstrating their presence in fluids or cell cultures and not in tissue. From the information available [Roche Diagnostics] a preliminary and novel technique was developed with the intention of creating a DNA probe for the detection of anti-ssDNA and anti-dsDNA antibodies *in situ.* If successful, this method would not only be able to demonstrate the presence of such antibodies, but would also show their actual location within the tissue. The latter being of particular interest in this project due to the possible pathological role of these antibodies in MS. The theory behind this method was that if DAPI was allowed to bind to ssDNA or dsDNA, when incubated with sections of tissue any anti-ds or -ssDNA antibodies present would bind to the probes and could then be visualised using fluorescence.

DAPI was formulated in deionised water at a stock concentration of 1 mg/mL. For the staining procedure, DAPI was diluted from the stock concentration, using methanol to give a concentration of 2 μg/mL. When combined with ssDNA or dsDNA a final concentration of 1 μg/mL of DAPI and 1 μg/mL DNA was obtained. DAPI and DNA were thoroughly mixed and incubated at room temperature in the dark for 1 hour prior to use.

Lewis rat spinal cord sections were prepared from an untreated rat and from a rat killed at the time of peak EAE. Sections were fixed in approximately 200 mL of acetone for 10 minutes following at least 2 hours of air-drying and were then left overnight to air dry.

The following day, spinal cord sections were washed with Dulbecco's buffer for 10 minutes prior to staining. At least 200 μL of the appropriate stain was added to each section as follows:

- Spinal cord from an untreated rat – DAPI alone
- Spinal cord from an untreated rat – DAPI bound to ssDNA
- Spinal cord from an untreated rat – DAPI bound to dsDNA

The above sections were included in order to determine if DAPI alone or as a DNA probe could cause unspecific staining, which would obscure any positive staining.

- Spinal cord from a rat sampled at peak EAE - DAPI alone

The above section was included to determine if DAPI alone could caused specific staining, which could lead to the presence of false positives.

- Spinal cord from a rat sampled at peak EAE – DAPI bound to ssDNA
- Spinal cord from a rat sampled at peak EAE – DAPI bound to dsDNA

The above sections were included as potential positive controls. Spinal cord from a rat sampled at peak was used, as based on previous work (IgG, IgM, B cell and C3 determination) this appeared to be the most likely time when anti-DNA antibodies would be present.

The slides were incubated at room temperature in the dark for 1 hour and then washed with Dulbecco's buffer for 10 minutes. The sections were embedded in Dako fluorescent mounting medium and allowed to dry. The slides were stored cool (nominally 2-8°C) overnight. Slides were read using a fluorescent microscope (Leica DMLB). A Jenopik ProgRes C14 camera was also used. DAPI has a peak excitation wavelength of 340 nm and an emission maximum at a wavelength of 488 nm. However, when bound to dsDNA the excitation peak occurs at a longer wavelength of approximately 360 nm. In this project fluorescence was visualised under green light for reasons discussed later on.

Following the initial work performed using DAPI probes, further work was conducted in order to refine the staining procedure and to gain confidence

in the results obtained. The following summarises the key changes to the method detailed above.

In addition to incubating ds and ssDNA with DAPI for 1 hour at room temperature in the dark, the mixture was also incubated for 1 hour in the dark under cool conditions. Various incubation times with tissue sections (1, 4 and 24 hours) were examined both at room temperature and under refrigerated conditions. DAPI concentrations of 1, 10, 100 and 1000 μg/mL were used and a DNA concentration of 10 μg/mL was also assessed.

The optimal conditions were found to be DAPI/DNA at 1 μg/mL (1:1 ratio), when allowed to incubate for 1 hour at room temperature in the dark. The incubation time with tissue sections was optimal when stored overnight refrigerated and in the dark.

In order to further increase the chance of detecting anti-dsDNA antibodies, rat spinal cord sections taken at the time of onset EAE were used. It was thought that by the time EAE had reached a peak, fewer anti-dsDNA antibodies may remain in the tissue. Whereas at the time of onset, when the disease is active, it is reasonable to think that the antibodies would be most prevalent. Onset samples were found to be no better than those taken at the time of peak EAE.

In order to minimise non-specific staining due to unbound DAPI in the probe mixture, a spin column was used to 'clean–up' the probe. The DAPI/DNA mixture was allowed to incubate for 1 hour at room temperature in the dark. The spin column 1000 (batch number 87H0612, Sigma) was inverted several times in order to suspend the matrix uniformly. The column was held upright and the seal broken. The column was placed in a 2 mL tube with the cap removed and centrifuged for 5 minutes at 700 g (1970 rpm). The collection tube was disposed of and the spin column was placed in a new 2 mL tube. A 70 µL DAPI/DNA sample was placed into the centre of the matrix bed's flat surface and the column was centrifuged for 5 minutes at 700 g. The spin column was disposed of leaving the purified sample in the bottom of the collection tube. A 50% recovery was assumed (based on the technical information supplied by Sigma) and based on this the purified sample was re-suspended in Dulbecco's buffer to provide a concentration of 10 µg/mL.

In order to investigate and substantiate potential positive staining, a dual staining technique was used. Serial sections of spinal cord obtained from a Lewis rat sampled at onset of EAE were stained for IgG, B cell or endothelium. A second set of sections was stained as before, but followed by dsDNA/DAPI probe and a third set was first stained with dsDNA/DAPI probe, followed by IgG, B cell or endothelium. Sections were also stained with dsDNA/DAPI probe followed by H & E in order to visualise the morphology of the sections.

2.4.10 Alexa Fluor 647 staining

Due to an absence of a convincing positive response, the DAPI stain was replaced with Alexa Fluor 647. The Alexa Fluor 647 kit was used to prepare dsDNA probes as follows.

One vial of DNase I was spun at 2100 rpm for 3 minutes to deposit the solid in the bottom of the tube. 100 µL of chilled DNase I storage buffer was added to the vial of DNase I to provide a concentration of 1 µg/µL and was stored frozen at nominally -20º prior to use.

The ULS reagent stock solution was prepared by adding 100 µL of 50% DMSO to the ULS reagent. The mixture was vortexed and stored cool (nominally 2 to 8ºC).

In order to obtain fragments of DNA (1 µg) of approximately 100 to 1000 base pairs in length the manufactures DNase protocol was followed. 1 µL of DNase stock (1 mg/mL) was diluted with 49 µL of 1x DNase I reaction buffer (20 µL of 10x DNase I reaction buffer in 180 µL of nuclease-free water, kept on ice). 3 µL of the DNase stock solution (1:50 dilution) was diluted with 1x DNase I reaction buffer to give a 1:1650 dilution.

1 mg/mL dsDNA was diluted with TE buffer to give a 100 µg/mL solution. The tube was flicked to mix and left on ice prior to use.

The following was added to a microtube; 9.5 µL nuclease-free water, 2.5 µL of 10x DNase I reaction buffer, 10 µL of 0.1 mg/mL sample DNA in TE buffer (10 mM Tris, 1 mM EDTA, pH 8), 3 µL DNase I working solution (1:1650). The mixture was allowed to incubate to 10 minutes at 37ºC after which the reaction was stopped by plunging the mixture into wet ice. The mixture was stored frozen prior to use.

To prepare the probe, DNA was precipitated by adding 2.5 µL of 3M sodium acetate, pH 5.2 (batch 112K8928, Sigma) and 50 µL of absolute ethanol (batch 11K3657, Aldrich) to 25 µL of DNA (1 µg). The mixture was frozen at -70ºC for 30 minutes and then centrifuged for 15 minutes at 12000 rpm. The resulting pellet was washed with 70% ethanol and allowed to air dry. The pellet was re-suspended in 20 µL of the labelling buffer. The DNA was denatured at 95ºC for 5 minutes and snap-cooled on wet ice. The mixture was centrifuged briefly at 2100 rpm for 3 minutes to redeposit the sample to the bottom of the tube. 5 µL of ULS labelling reagent stock was added to the denatured DNA. The mixture was incubated at 80ºC for 15 minutes and the reaction stopped by plunging into an ice bath. The mixture was centrifuged briefly at 2100 rpm for 3 minutes to redeposit the sample to the bottom of the tube.

Purification of the probe was performed using a QIAquick spin PCR purification kit. 24 mL of ethanol was added to PE buffer, 500 µL of which was then added to 100 µL of DNA sample. The spin column was placed in a 2 mL tube and the DNA sample placed in the spin column and

centrifuged at 13000 rpm for 45 seconds. The flow was discarded and the column replaced. 3 mL of buffer PE was added to the spin column which was then centrifuged for 45 seconds. The flow was discarded, the spin column replaced in the tube and was then centrifuged for 1 minute. The spin column was placed in a clean 1.5 mL microcentrifuge tube. DNA was eluted by adding 120 µL of buffer EB to the centre of the membrane. After being left to stand for 1 minute, the mixture was centrifuged for 1 minute. An appropriate volume of Dulbecco's buffer was added to the ULS/DNA probe mixture in order to obtain the required concentration for use.

Alexa Fluor 647 has an excitation wavelength of 647 nm and an emission wavelength of 665 nm. As such tissue sections incubated with the Alexa Fluor/dsDNA probe were viewed under red light (wavelength approximately 650 nm).

Following four Alexa Fluor probe preparations and in the absence of positive staining, the quality of the probe preparation was questioned. Probe preparation was subsequently prepared by a contact at United Leeds Teaching Hospital.

2.5 Method Validation of the Alexa Fluor Probe for Use in Anti-dsDNA Antibody Demonstration in Biozzi (ABH) Mouse CNS

2.5.1 Materials

Plasmid dsDNA, 1 mg/mL (lot 5) was supplied by Bayou Biolabs, USA. Alexa Fluor 647 was supplied by Molecular probes Europe BV, The

Netherlands. Alexa Fluor/dsDNA probes were prepared at United Leeds Teaching Hospital.

2.5.2 Sectioning

5 micron sections of Biozzi (ABH) mouse spinal cord were sectioned at 50 micron intervals using a cryostat. Sections were placed on APES coated slides. Slides were stored frozen (nominally -70ºC) prior to staining.

2.5.3 Determination of anti-dsDNA antibodies using Alexa Fluor/dsDNA probes

Six spinal cord sections from Biozzi (ABH) mice (three from each mouse) which had been killed at peak EAE during a second episode of the clinical signs were removed from the freezer and allowed to air dry overnight.

The following day the sections were placed in acetone for 10 minutes and then allowed to air dry for approximately 30 minutes. The slides were washed with Dulbecco's buffer for 10 minutes and then placed in slide holders and washed again for 10 minutes with Dulbecco's buffer. At least 100 µL of Alexa Fluor/dsDNA was added to four of the spinal cord sections (two from each mouse). The remaining two sections were incubated with Dulbecco's buffer only and served as negative controls. The slides were placed in the refrigerator, protected from light and were allowed to incubate for approximately 4 hours. The slides were removed from the fridge and washed for 10 minutes with Dulbecco's buffer. The slides were removed from the slide holders and mounted using Dako immuno-fluorescence mounting medium and allowed to dry.

Biozzi (ABH) mouse and Lewis rat brain sections, and Lewis rat spinal cord sections were also stained in this way.

2.5.4 Dual staining with Alexa Fluor/dsDNA probe and H & E

In order to investigate the apparent auto-fluorescence seen in spinal cord sections taken from Biozzi (ABH) mice exhibiting EAE, dual staining with the dsDNA probe and H & E was performed. For this purposes spinal cord sections were stained as follows.

1. Serial sections taken from an untreated Biozzi (ABH) mouse:-

- 2 x spinal cord – Dulbecco's buffer
- 2 x spinal cord – H & E
- 2 x spinal cord – Alexa Fluor/dsDNA probe

The sections above were stained in order to see if auto-fluorescence could be seen in the spinal cord of untreated mice. These sections were compared with the negative control Dulbecco's buffer stained sections. The H & E sections were included for morphological purposes.

2. Serial sections taken from a Biozzi (ABH) mouse sampled at peak EAE during the second episode of clinical signs:-

- 2 x spinal cord - Alexa Fluor/dsDNA probe
- 2 x spinal cord - H & E
- 2 x spinal cord - Alexa Fluor/dsDNA probe followed by H & E
- 2 x spinal cord - H & E followed by Alexa Fluor/dsDNA probe

Various combinations of H & E and Alexa Fluor/ds DNA probe were used in order to identify and correlate areas of fluorescence.

The most successful method of dual staining was found to be staining the sections initially with the dsDNA probe and then looking for areas of fluorescence under the microscope. Once fluorescence had been detected the co-ordinates of this were recorded and the sections re-stained with H & E for (approximately 1 minute). The slide was then re-aligned with co-ordinates and examined.

2.6 Immuno-histological Staining of Human Tissue

2.6.1 Introduction

In order to investigate the ability of Alexa Fluor/dsDNA probes to detect anti-dsDNA antibodies it was necessary to obtain human brain and spinal cord from an individual who had multiple sclerosis at the time of their death. Although the detection of anti-dsDNA antibodies in these tissue sections could not be guaranteed, published literature [Williamson *et al*. (2001)] supports the presence of these antibodies around active plaques in multiple sclerosis. This is in contrast to rat and mouse EAE models for which no such literature could be found referencing anti-dsDNA antibodies *in situ*. A request was made to the Multiple Sclerosis Tissue Bank for sections taken from the brain and spinal cord which were exhibiting plaque activity at the time of death. Sections obtained from the Tissue Bank had been cut from a cerebral tissue block, a couple of these sections were stained with LFB (Luxol fast blue)/MHC class II, which revealed the presence of a large lesion with immune activity on the lesion edge. Luxol fast blue is a stain used to demonstrate demyelination and can also enable phagocytic macrophages containing myelin basic protein to be

visualised. MHC class II staining was used to demonstrate MHC class II molecule expression. In addition, sections of human spinal cord taken from a patient with MS were supplied. Information provided by the Tissue Bank indicated that staining of the spinal cord block had been found to be less clear. Although there appeared to be an area of myelin pallor (depleted myelin) that corresponded to an elevated MHC class II expression, the lesion was not easily identified.

2.6.2 Materials

Fifteen brain and spinal cord flash frozen sections were supplied by the UK MS Tissue Bank, on dry ice. Sections were taken from a 51 year old male who had been suffering from multiple sclerosis for approximately 10 years. The classification of MS was secondary progressive multiple sclerosis, which is a steadily progressive form of the disease which may follow many years of relapsing/remitting MS. In general, secondary progressive multiple sclerosis is associated with lower levels of inflammatory lesion formation than that seen in relapsing/remitting MS. The cause of death was sclerosis and bronchopneumonia. His mother also had MS.

Plasmid dsDNA, 1 mg/mL (lot 5) was supplied by Bayou Biolabs, USA. Alexa Fluor 647 was supplied by Molecular probes Europe BV, The Netherlands. Monoclonal mouse anti-human plasma cell (Clone VS38c), batch 00005455, supplied by DakoCytomation. Albumin bovine serum (batch 103K1363) supplied by Sigma, UK. Hydrogen peroxide (batch 123K3250) supplied by Sigma, UK. Rabbit serum (batch R0511) supplied

by Vector Laboratories Inc., USA. Rabbit anti-mouse Immunoglobulins – biotinylated (batch 00015064) supplied by Dako, Denmark. StreptABC Complex/HRP (batch 00012270) supplied by Dako, Denmark. Avidin/Biotin (batch R0527) supplied by Vector Laboratories Inc, USA. Alexa Fluor/dsDNA probes were prepared at United Leeds Teaching Hospital.

2.6.3 Sectioning

5 micron sections of human tonsil were sectioned at 50 micron intervals using a cryostat, sections were and placed on APES coated slides. Slides were stored frozen (nominally -70ºC) prior to staining.

2.6.4 Determination of plasma cells - validation

Due to the elusive anti-dsDNA antibodies, the presence of plasma cells in human CNS tissue sections was investigated. The presence of such cells would indicate that immune activity was occurring and that it should be possible to detect anti-dsDNA antibodies in the serial sections taken if present.

Human tonsil and spleen sections were removed from the freezer and allowed to air dry overnight. Tonsil and spleen were used to validate plasma cell demonstration as such cells reside in abundance in these organs.

The following day, the slides were placed in approximately 200 mL of acetone for 10 minutes to fix the sections and for approximately 1 hour at

room temperature to air dry. The slides were then washed with Dulbecco's buffer for 10 minutes. Slides were blocked for endogenous enzyme, using 750 µL of hydrogen peroxide in 200 mL methanol (0.1125%) for 30 minutes. Slides were then washed with running tap water for 10 minutes and returned to Dulbecco's buffer for washing with Dulbecco's buffer for 10 minutes. Slides were serum blocked by adding at least 100 µL of 1:5 rabbit serum in Dulbecco's buffer for 20 minutes. The slides were then washed with Dulbecco's buffer for 10 minutes. 100 µL of avidin was added for 15 minutes, the slides were washed with Dulbecco's buffer for 10 minutes and Biotin (to prevent avidin non-specific binding) was added for a further 15 minutes. Following washing with Dulbecco's buffer for 10 minutes, at least 200 µL of the primary antibody, monoclonal mouse anti-human plasma cell in 2% bovine serum albumin at dilutions of 1:25, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, were added to each slide as appropriate. One section of tonsil and spleen remained as negative controls and as such had at least 200 µL of 2% bovine serum albumin added to them. The slides were left at room temperature for approximately 90 minutes. The slides were washed with Dulbecco's buffer for 10 minutes. At least 100 µL of StreptABCComplex/HRP was added for approximately 45 minutes after which the slides were washed for 10 minutes with Dulbecco's buffer. The slides were removed from the slide holders and placed in Dulbecco's buffer. Slides were placed in the chromogen DAB for 10 minutes. DAB was prepared immediately prior to use by adding 0.5 g of DAB to 1 litre of buffer containing 1 mL of 30% hydrogen peroxide solution. Once removed from DAB the slides were

washed in running tap water for 5 minutes. The slides were then placed in the chromogen enhancer, copper sulphate for 5 minutes, following which the slides were washed in running tap water for 5 minutes. The slides were counter-stained with haematoxylin for 1 minute, rinsed briefly in tap water and placed in Scott's tap water for 1 minute and finally briefly rinsed in tap water.

2.6.5 Validation of plasma cell determination

Plasma cell determination was validated using various dilutions of the primary antibody. Dilutions (1:25, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600) of monoclonal mouse anti-human plasma cell (Clone VS38c) were used on sections of human tonsil. In addition, a section of tonsil and spleen were included as negative controls, i.e. 2% bovine serum albumin was added in place of the primary antibody.

On the basis of the results obtained a dilution of 1:80 was considered appropriate for subsequent staining (figures 9 and 10). This staining method was never used on any other tissue sections due to the subsequent positive staining of anti-dsDNA antibody and the limited number of human tissue samples available.

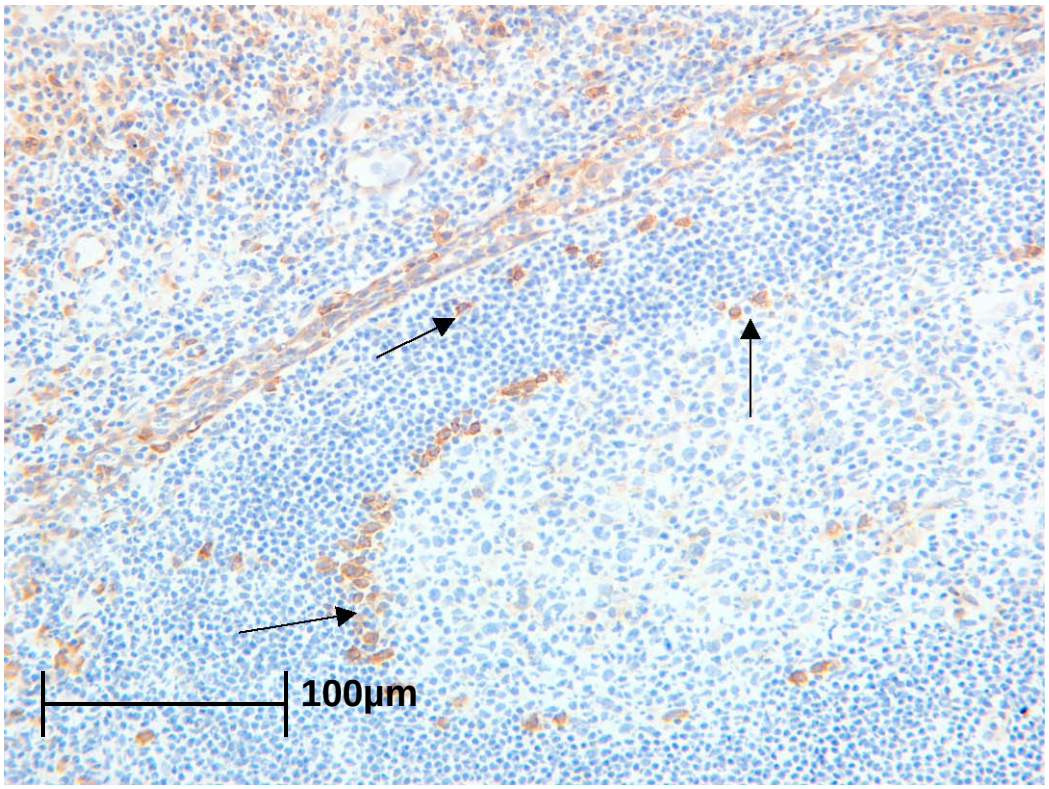

Figure 9 Plasma cell staining (1:80 dilution) using monoclonal mouse anti-human plasma cell and counter stained with haematoxylin, under light microscopy (x 20 objective). The arrows point to areas of positive staining on a human tonsil section.

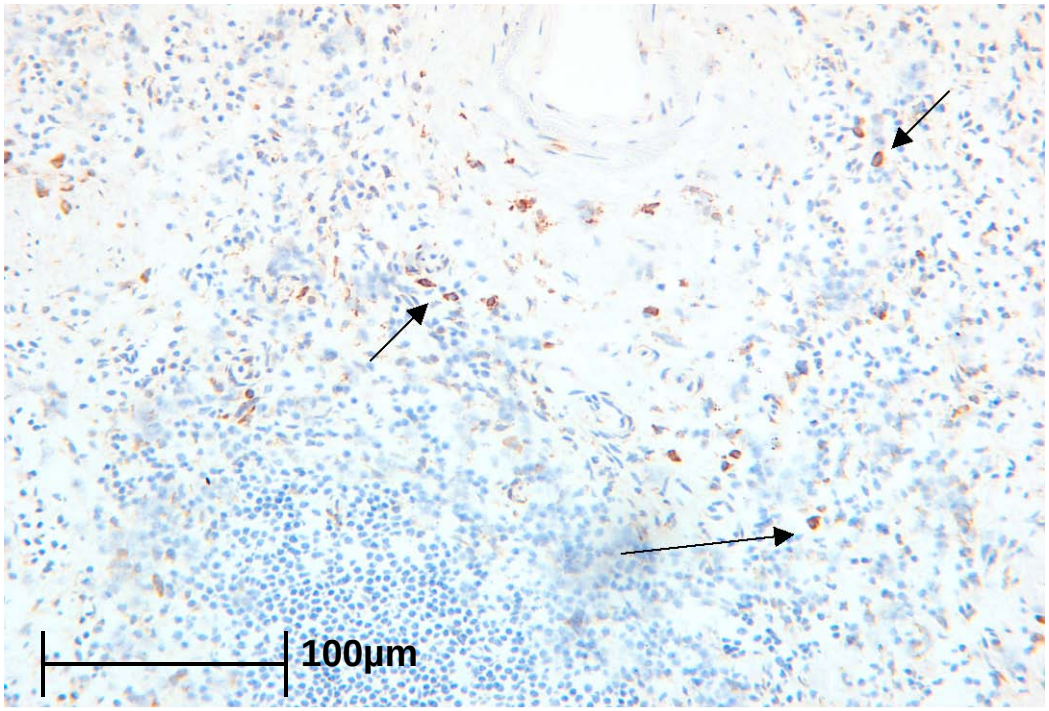

Figure 10 Plasma cell staining (1:80 dilution) using monoclonal mouse anti-human plasma cell and counter stained with haematoxylin, under light microscopy (x 20 objective). The arrows point to areas of positive staining on a human spleen section.

2.6.6 Determination of anti-dsDNA antibodies using Alexa Fluor/dsDNA probes

Sections of MS human brain and spinal cord were allowed to air dry overnight.

The following day the sections were placed in acetone for 10 minutes and then allowed to air-dry for approximately 30 minutes. The slides were washed with Dulbecco's buffer for 10 minutes and then placed in slide holders and washed again for 10 minutes with Dulbecco's buffer. At least 100 µL of undiluted probe was added to each slide, with the exception of one brain and one spinal cord section to which Dulbecco's buffer was added, these sections served as negative controls. The slides were placed in the refrigerator, protected from light for approximately 4 hours. Slides were removed from the fridge and washed for 10 minutes with Dulbecco's buffer. The slides were removed from the slide holders and mounted using Dako immuno-fluorescence mounting medium and allowed to dry.

The Alexa Fluor probe was used undiluted due to lack of staining when diluted.

3 CHAPTER 3: RESULTS

3.1 Clinical signs of EAE in the Lewis rat– in vivo phase

Following inoculation with the encephalitogenic agent, the majority of animals displayed signs of EAE, some animals however displayed signs to a greater severity than others. This led to the animals being categorised into two groups, successful and unsuccessful. Animals deemed to be successful displayed an anticipated EAE response at the time of sampling as follows;

- Onset – animals achieving a score of 1 (slightly limp tail) by Day 10.
- Peak – animals achieving a score of 3 or more by Day 10.
- Plateau – animals achieving a score of at least 6.
- Remission – animals achieving a score of at least 6.
- Relapse (Day 34) - animals achieving a score of at least 6.

The term peak relates to the approximate time at which the inflammatory response was expected to have reached peak activity. It was considered important to sample the animals before the disease reached a plateau as at this time it was hypothesised that the inflammatory response could be coming to an end. For this reason the score for clinical signs was seen to be higher in the plateau group compared to the peak sample group. In general, histopathological evaluation found there to be a greater presence of inflammatory deposits in animals sampled at peak compared to those sampled at plateau.

Animals deemed unsuccessful displayed EAE signs to a lesser extent than that stated above or failed to exhibit signs. The first occurrence of signs following inoculation was also delayed in some cases when compared to the successful animals.

The times of sampling (CSF, blood, spinal cord and brain) were at;

- Onset - the time at which the first signs were seen.
- Peak - the time at which a substantially limp tail was observed.
- Plateau - the time at which the same signs had been seen on at least two consecutive occasions.
- Remission - at the time when the animals had fully recovered.
- Relapse - which for the Lewis rat was Day 34 due to relapse not occurring in this particular model of EAE.

Although relapse was not anticipated in the Lewis rat EAE model, this sample time-point was included to assess any potential histopathological changes in the absence of EAE clinical signs.

Figures 11a and 11b show the difference between group mean total EAE scores at the time of sampling for successful and unsuccessful animals, respectively. The differences in EAE development were as follows;

- Although animals in the onset group had the same total score, the unsuccessful animals were slower in developing the signs; Day 11 compared to Day 9 and 10 for successful animals.
- At peak a maximum score 3 or 7 was exhibited by successful animals compared to scores of 0, 1 or 2 for unsuccessful rats.
- At plateau a maximum EAE score of 6 or 7 was exhibited by successful animals compared to scores of 1 or 2 for unsuccessful rats.
- At remission and relapse (Day 34), successful rats had maximum EAE scores of 6 or 7 compared to 1 or 2 for unsuccessful rats.

Thirty-nine out of 40 rats immunised with CNS material in the presence of an adjuvant (carbonyl iron), displayed signs of EAE to some extent. All 16 control (carbonyl iron) rats and four untreated rats appeared normal throughout the study.

3.1.1 Onset EAE group

All eight rats in the onset group were killed on the appearance of a slightly limp tail (EAE score of 1). EAE was first apparent in one rat on Day 9, with all rats killed and sampled by Day 11. For the data analysis, the four rats that exhibited signs by Day 10 were treated as successful as they matched the selection criteria, whereas the four rats not showing signs until Day 11 were classed as unsuccessful in EAE development.

Although the time difference between the development of successful and unsuccessful signs was negligible, a decision was made at the time to distinguish between these two groups of animals to see if there was any

difference histopathologically. The problem with sampling this group of animals was that there was no way of knowing that if left alone, what the maximum severity score would be. That is, whether the animals were truly successful or unsuccessful. Results from this study showed there to be no correlation between the time to EAE onset and the successfulness or unsuccessfulness of the animals to develop EAE clinical signs.

3.1.2 Peak EAE group

Seven out of eight rats in this group displayed signs of EAE to some extent. Rats in the peak EAE sampling group were killed on the first day that they exhibited a substantially limp tail. Only five rats in this group were classed as successful, with four of these rats having an EAE score of 3 (substantially limp tail) and one having a maximum score of 7 (ataxia and a substantially limp tail). EAE was first seen in two rats on Day 8 with all successful animals killed and sampled by Day 10. There were three unsuccessful rats in this group, two of which had a moderately limp tail as a maximum sign; one rat failed to show any indication of EAE.

3.1.3 Plateau EAE group

All eight rats in the plateau group exhibited signs of EAE. Five out of eight rats were killed after achieving a maximum score of at least 6, with the majority of these animals exhibiting a substantially limp tail and ataxia for two consecutive days. EAE was first apparent in one rat on Day 9, with a plateau occurring between Day 12 and Day 16. The remaining rats in this group exhibited slight signs of EAE with a score of no more than 2

(moderately limp tail), these rats were treated as unsuccessful in developing EAE.

3.1.4 Remission EAE group

All rats (including those deemed unsuccessful) in the remission group displayed signs of EAE to some extent in this study and when killed had an EAE score of 0 (normal). The first signs of EAE were initially seen in four rats on Day 10, with three out of the eight rats exhibiting marked signs of EAE (at least a score of 6) on Day 15 or 16. These three rats were regarded as successful. The five unsuccessful rats displayed slight EAE signs with scores of no more than 2 (moderately limp tail). The majority of animals appeared normal on Day 18 and by Day 20 all rats had been killed and sampled.

3.1.5 Relapse EAE group (Day 34)

As anticipated, rats in this study did not show any indication of relapse and as such were killed and sampled on Day 34. All rats in this group developed EAE, with three out of the eight rats having a peak score of at least 6, as such these rats were treated as successfully developing EAE. All other rats in this group had a peak score of 2. EAE was first apparent in all but one rat on Day 10, peak EAE was observed on Day 15 or 16, and all but two rats appeared normal by Day 22. At the time of kill (Day 34) two rats had an EAE score of 1 (slightly limp tail) all other rats appeared normal.

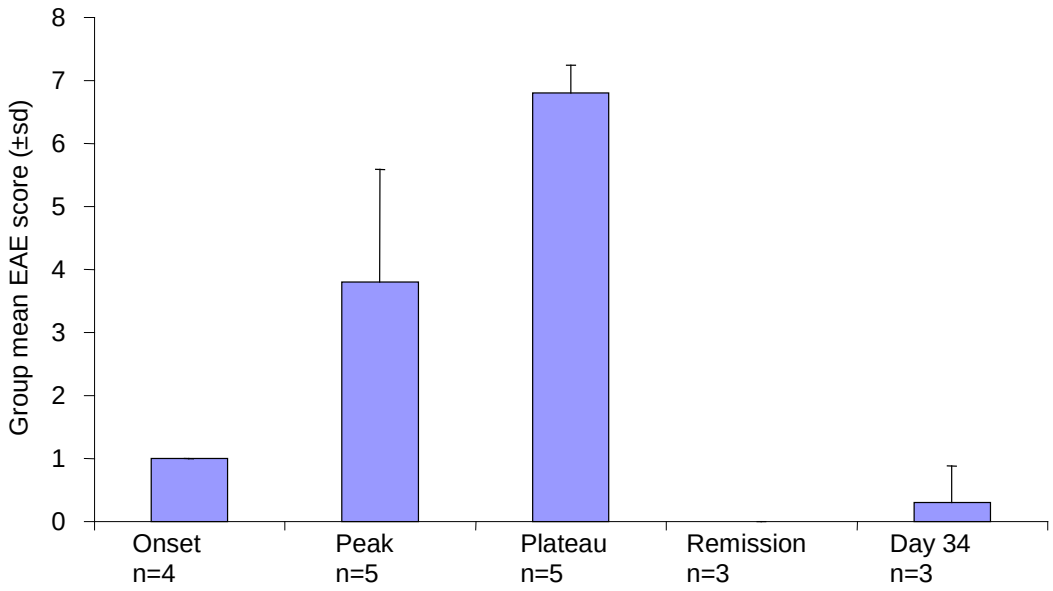

Figure 11a

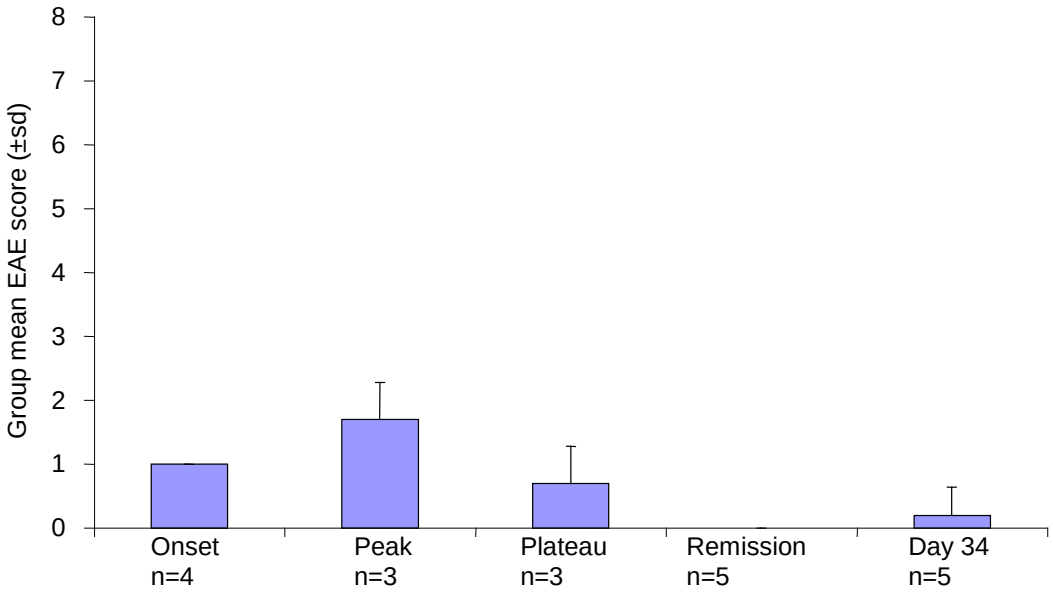

Figure 11b

Figure 11 Bar graphs showing (a) Group mean total EAE scores in successful rats at the time of sampling for animals immunised with CNS material. (b) Group mean total EAE scores in unsuccessful rats at the time of sampling for animals immunised with CNS material. EAE signs were absent in all control (carbonyl iron) animals.

3.2 Detection of anti-ss and -dsDNA antibodies in Lewis rat CSF and sera using ELISA

Cerebrospinal fluid and sera samples obtained from Lewis rats used in the EAE model, killed and sampled at EAE onset, peak, plateau, remission or 'relapse' were analysed using an ELISA at a dilution of 1:50 and where volumes permitted, undiluted.

The results are summarised in Tables 4 and 5.

Group	Samples taken at EAE point	Group mean OD (±se) using an ELISA for anti-dsDNA antibody			
		CSF		Sera	
		1:50	Undiluted	1:50	Undiluted
	Untreated	0.00$^{(1)}$ ±0.000	0.11$^{(2)}$ ±0.021	0.00$^{(4)}$ ±0.000	0.12$^{(4)}$ ±0.019
Part II (peak)	Untreated	Not tested	Test failed	Not tested	Not tested
2a	Onset	0.01$^{(7)}$ ±0.008	0.11$^{(5)}$ ±0.009	0.00$^{(8)\dagger\dagger}$ ±0.000	0.11$^{(8)**}$ ±0.004
2e	Peak	Not tested	Test failed	Not tested	Not tested
2b	Plateau	0.01$^{(8)}$ ±0.007	0.26$^{(4)}$ ±0.053	0.01$^{(8)}$ ±0.006	0.12$^{(6)}$ ±0.015
2c	Remission	0.00$^{(6)}$ ±0.000	0.12$^{(5)}$ ±0.026	0.02$^{(5)}$ ±0.013	0.13$^{(8)}$ ±0.029
2d	Relapse	0.01$^{(4)}$ ±0.005	0.16$^{(6)\dagger}$ ±0.047	0.02$^{(5)}$ ±0.015	0.11$^{(7)}$ ±0.009
3a	Carbonyl control – onset	0.00$^{(2)}$ ±0.000	0.14$^{(2)}$ ±0.035	0.00$^{(2)}$ ±0.000	0.08$^{(4)}$ ±0.002
3e	Carbonyl control – peak	Not tested	Test failed	Not tested	Not tested
3b	Carbonyl control – plateau	0.00$^{(2)}$ ±0.000	0.20$^{(2)}$ ±0.099	0.02$^{(4)}$ ±0.010	0.06$^{(3)}$ ±0.024
3c	Carbonyl control – remission	0.00$^{(3)}$ ±0.000	0.10$^{(2)}$ ±0.001	0.00$^{(4)}$ ±0.000	0.10$^{(4)}$ ±0.006
3d	Carbonyl control - relapse	0.00$^{(4)}$ ±0.000	0.10$^{(1)}$ ±0.000	0.00$^{(3)}$ ±0.001	0.11$^{(4)}$ ±0.006

Table 4 Group mean OD values (±se) using an anti-dsDNA antibody ELISA.

The numbers in parentheses indicate the number of samples analysed.
Group means exclude samples with a CV >25%. † not calculable due to only one value.
†† not calculable due to 'perfect' data.
Statistical significance of difference from the associated carbonyl group using Wilcoxon two sample test: **P<0.01.

Group	Samples taken at EAE point	Group mean OD (±se) using an ELISA for anti-ssDNA antibody			
		CSF		Sera	
		1:50	Undiluted	1:50	Undiluted
	Untreated	All samples >25% CV	No sample	Test failed	0.13(1) ±0.000
Part II (peak)	Untreated	Not tested	Not tested	Not tested	Not tested
2a	Onset	0.49(2) ±0.102	No sample	Test failed	0.13(1) † ±0.000
2e	Peak	Not tested	Not tested	Not tested	Not tested
2b	Plateau	0.11(1) † ±0.000	No sample	Test failed	0.21(4) † ±0.049
2c	Remission	0.66(2) † ±0.149	No sample	Test failed	0.19(3) † ±0.057
2d	Relapse	0.67(2) ±0.094	No sample	Test failed	0.16(4) † ±0.014
3a	Carbonyl control – onset	0.29(2) ±0.124	No sample	Test failed	0.12(3) ±0.026
3e	Carbonyl control – peak	Not tested	Not tested	Not tested	Not tested
3b	Carbonyl control – plateau	0.09(2) ±0.006	No sample	Test failed	0.15(1) ±0.000
3c	Carbonyl control – remission	0.11(1) ±0.000	No sample	Test failed	All samples >25% CV
3d	Carbonyl control - relapse	0.10(4) ±0.011	No sample	Test failed	All samples >25% CV

Table 5 Group mean OD values (±se) using an anti-ssDNA antibody ELISA.

The numbers in parentheses indict the number of samples analysed.
Group means exclude samples with a CV >25%. † not calculable due to only one value
Statistical significance of difference from the associated carbonyl group using Wilcoxon two sample test: P>0.05.

3.2.1 Detection of anti-dsDNA antibodies in Lewis rat CSF

No anti-dsDNA antibodies were detected in diluted CSF samples from untreated, EAE or carbonyl control animals. CSF samples diluted by 1:50 and analysed in duplicate, were found to have an OD range of 0.00 to 0.01.

In order to confirm these results, where sufficient volumes of CSF allowed, undiluted CSF samples were analysed. Duplicate analysis was not possible due to insufficient quantities available.

The analysis of single samples of undiluted CSF was found to have an OD range of 0.11 to 0.26. Although the highest OD values were found in samples taken from rats sampled at the plateau of EAE, the results show the presence of anti-dsDNA antibody at extremely low levels and not significantly different from the associated carbonyl control group. In addition, the OD range for the carbonyl control groups was comparable to that of the EAE groups with an OD range of 0.10 to 0.20. The OD for untreated animal samples was 0.11.

3.2.2 Detection of anti-dsDNA antibodies in Lewis rat sera

No anti-dsDNA antibodies were detected in diluted sera samples. When analysed in duplicate, diluted sera from untreated, EAE and carbonyl control animals were found to have an OD range of 0.00 to 0.02.

In order to confirm these results, undiluted sera samples were analysed in duplicate. The OD obtained for these samples ranged between 0.11 and 0.13 for EAE rats. When compared to the associated carbonyl group, anti-dsDNA antibodies in undiluted sera taken from animals at the time of onset of EAE was found to be statistically significantly ($P<0.01$) increased. However, the OD at onset (0.11) was comparable to that of the untreated group of animals (OD 0.12) and as such is not considered to be biologically significant. An OD range of 0.06 to 0.11 was obtained for the carbonyl groups.

Undiluted CSF and sera obtained from Lewis rats in Part II of the rat EAE model, i.e. those sampled at peak EAE, were analysed separately. For reasons unknown, the standard response curve failed the acceptance criteria on the basis that a number of the calibration standards failed to be within 25% of the nominal concentrations. As such the results obtained were considered to be invalid. Due to insufficient quantities of CSF it was not possible to re-run the dsDNA assay or to perform the ssDNA analysis.

This problem (insufficient volumes of CSF) highlighted the need for finding an alternative method for detecting anti-dsDNA antibodies.

3.2.3 Detection of anti-ssDNA antibodies in Lewis rat CSF

In contrast to the anti-dsDNA antibody assay, the anti-ssDNA antibody ELISA of CSF (1:50) indicated that this antibody was present in the CSF of all EAE animals, with an OD range of 0.11 to 0.67. In general, a markedly lower OD was obtained for CSF samples from the control animals

(carbonyl iron-treated) with a range of 0.09 to 0.29. It was not possible to confirm these results using undiluted CSF, due to insufficient quantities available. All samples from untreated animals had a sample CV value of >25% and as such there were no meaningful results available for this group.

3.2.4 Detection of anti-ssDNA antibodies in Lewis rat sera

The OD range for undiluted sera from EAE rats was 0.13 to 0.21, with the highest value occurring at plateau. Carbonyl OD for undiluted samples was 0.12 to 0.15.

The response curve obtained for diluted sera samples (1:50) was considered to be unacceptable and of no value in this study for the following reasons. The lowest dilution of the response curve (1:40000) had an OD of 0.973, this being abnormally high. For the first set of reference standards the 1:250 dilution was lower than found previously (OD of 0.536). The intermediate (1:2000) and low (1:10000) dilutions of the reference standards at the end of the plate were higher than expected, with OD values of 0.951 and 0.896, respectively. As such the results for diluted sera analysed for anti-ssDNA antibodies are not discussed.

Although statistical analysis was performed on the ELISA data sets, this analysis is considered to be of little value due to the limited data available. However, when the data is presented graphically (figures 12-15) a distinct and opposing trend can be seen between anti-ssDNA and anti-dsDNA antibody levels in CSF. At the time of onset, anti-ssDNA antibody levels

are markedly (5-fold) higher than anti-dsDNA antibody levels, however by the time clinical signs have reached a plateau, anti-ssDNA antibody levels have fallen sharply below the levels of anti-dsDNA antibodies, which at this time-point show a 3-fold increase. By the time of remission, both antibody types have returned to levels comparable to those seen at onset.

Sera anti-ssDNA and anti-dsDNA antibody levels on the other hand appear to have remained relatively constant throughout the course of the disease process.

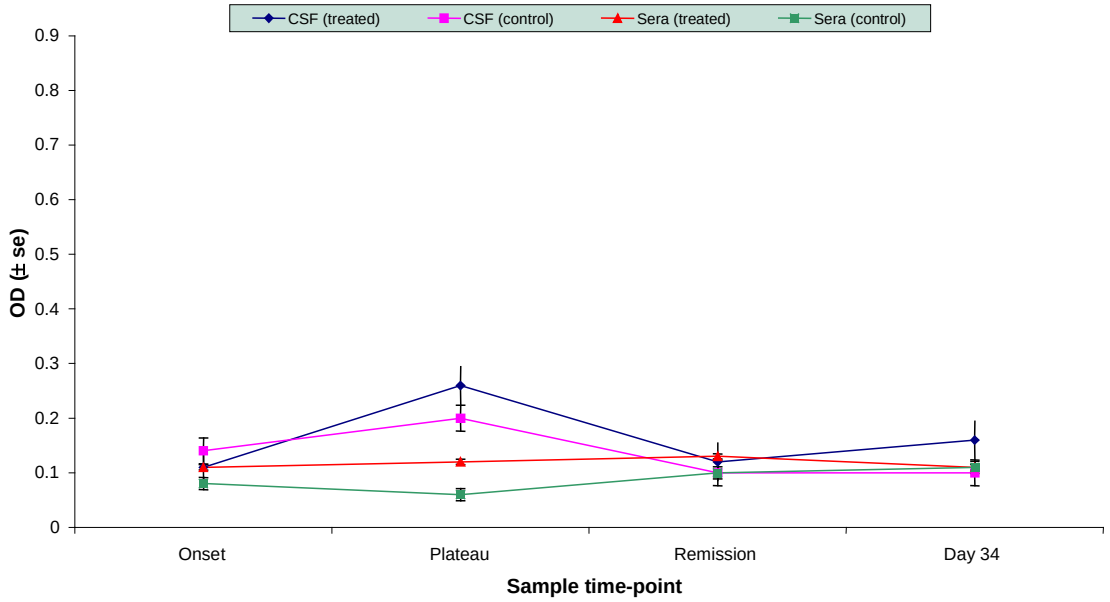

Figure 12 Anti-dsDNA antibody levels in undiluted sera and CSF from EAE or carbonyl control Lewis rats sampled at onset, plateau, remission or Day 34 (relapse).

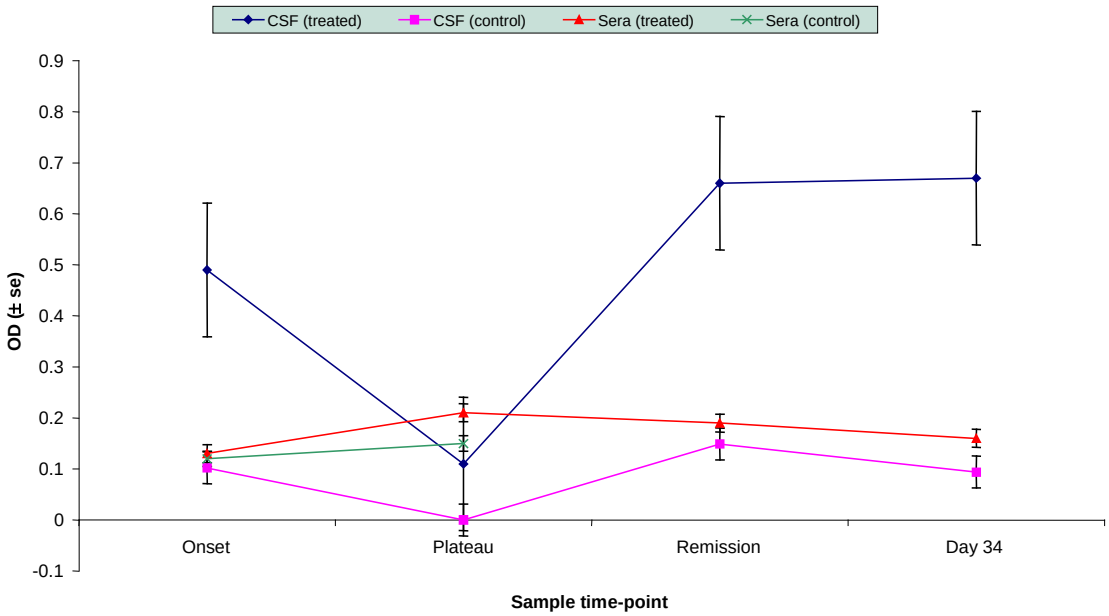

Figure 13 Anti-ssDNA antibody levels in sera (undiluted) and CSF (1:50 dilution) from EAE or carbonyl control Lewis rats sampled at onset, plateau, remission or Day 34 (relapse).

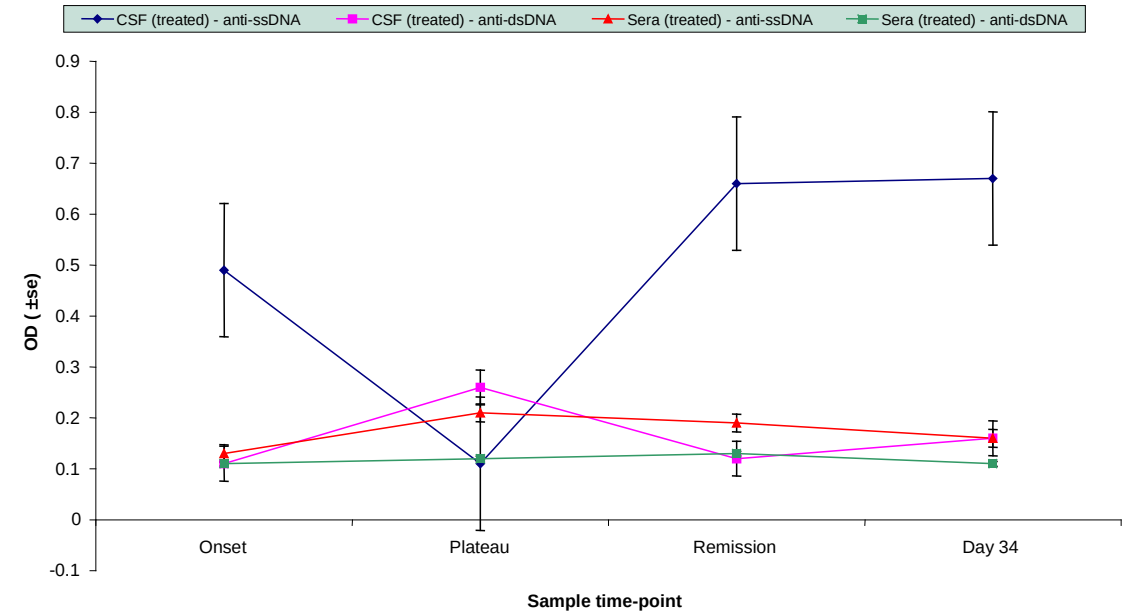

Figure 14 Anti-ssDNA versus anti-dsDNA antibody levels in undiluted sera and CSF (1:50 dilution for anti-ssDNA antibodies) from EAE Lewis rats sampled at onset, plateau, remission or Day 34.

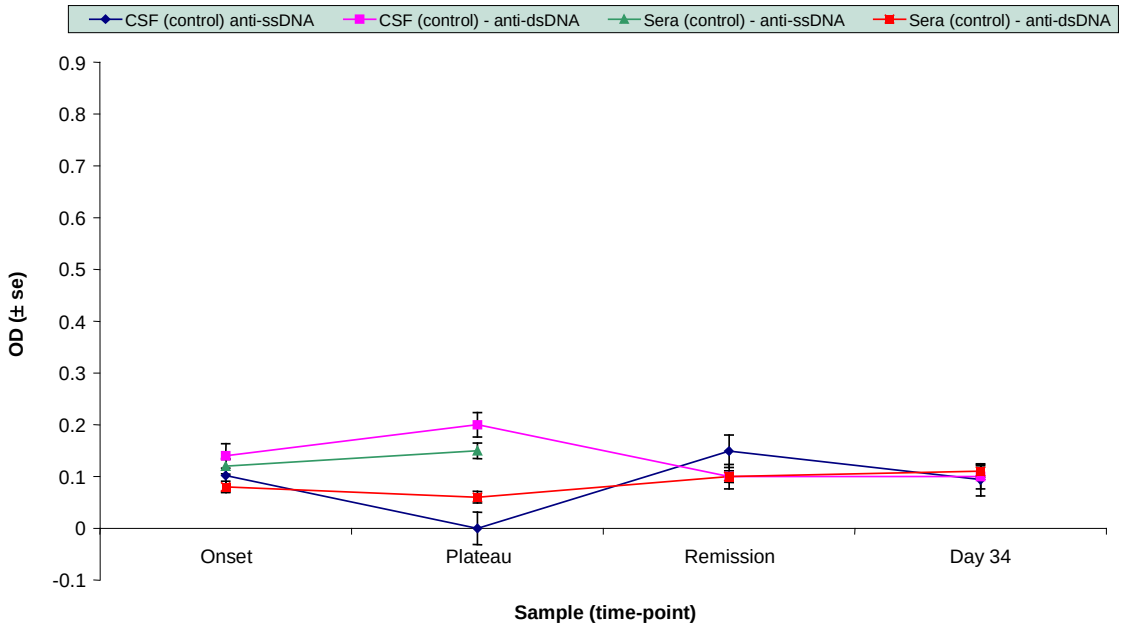

Figure 15 Anti-ssDNA versus anti-dsDNA antibody levels in undiluted control sera and CSF (1:50 dilution for anti-ssDNA antibodies) from carbonyl control Lewis rats sampled at onset, plateau, remission or Day 34.

3.3 Histopathology

Histological techniques were used to investigate the incidence of inflammatory deposits (C3, IgG, IgM and B cells) in sections taken from the central nervous system, in order to profile the disease process.

IgG and IgM antibodies were evaluated to see if the incidence of one was different to the other at particular time-points during the disease process. IgG antibody was of particular interest as high affinity anti-dsDNA antibodies are likely to be of this class. B cells were of interest with regards not only to their presence, but also to what they were secreting. C3 is important in the activation of the complement system and is important in the development of inflammation.

The incidence of each parameter (C3, IgG, IgM or B cells) was evaluated by examining two separate sections of spinal cord from each animal. From each section ten random areas were selected, i.e. 20 separate areas scored per rat. The presence distinct areas of positive staining was recorded (as a count) along with its location with regards to areas of perivascular cuffing. For this purpose the sections were viewed under the x 20 objective. The group mean was determined by adding the number of incidences of positive staining together and dividing by the total number of sections viewed for a particular group of animals (treatment / time-point / control / untreated / successful / unsuccessful).

3.3.1 C3 staining

Figure 16 shows C3 immunostaining under light microscopy (x40 objective). The section was taken from a female Lewis rat that had been immunised with CNS material and killed at peak EAE.

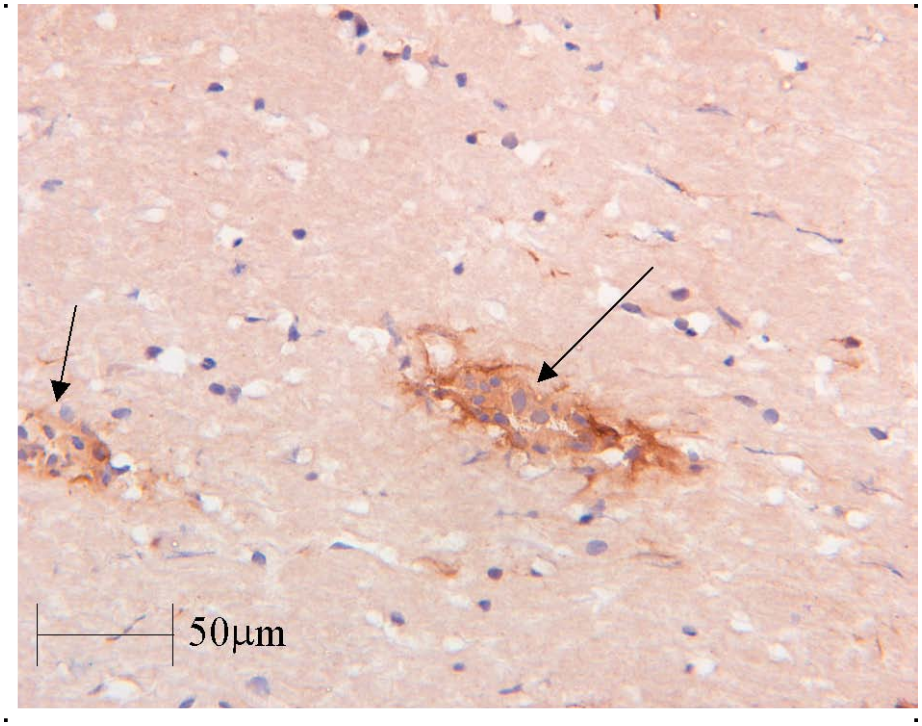

Figure 16 C3 staining using sheep anti-rat complement C3 and counter stained with haematoxylin, under light microscopy (x 40 objective). Arrows point to areas of positive C3 staining on a spinal cord section taken from a female Lewis rat that had been immunised with CNS material and killed at peak EAE.

Figures 17a-17c indicate that comparable total C3 staining was found at each time-point in spinal cord sections of animals showing EAE signs, animals failing to show acceptable signs of EAE and control animals. Minimal C3 was present in the spinal cord of untreated animals.

Specific staining at non-perivascular cuffing sites was also similar between successful and unsuccessful animals, with the greatest C3 staining apparent for animals sampled at peak EAE. In general, there was little difference in the incidence of C3 between the different time-point groups sampled, the exception being those sampled at peak EAE. When unsuccessful and successful animals were compared to the carbonyl control groups using a Wilcoxon two sample test there was no statistically significant difference.

C3 detection at the sites of perivascular cuffing in animals exhibiting EAE and to a lesser extent in those that failed to show comparable EAE signs, display a trend which increases from onset of EAE, reaching a maximum at peak EAE and diminishing thereafter. C3 incidence was approximately twice as high in animals showing EAE signs than in those showing minimal signs. C3 presence at perivascular cuffing sites in the control animals was found to be negligible in comparison. When unsuccessful animals were compared to the associated carbonyl control animals using a Wilcoxon two sample test there was no statistically significant difference. When EAE successful animals were compared to the associated control

animals using the Wilcoxon two sample test, the increase in C3 was found to be statistically significant ($P<0.001$) at the time of peak EAE.

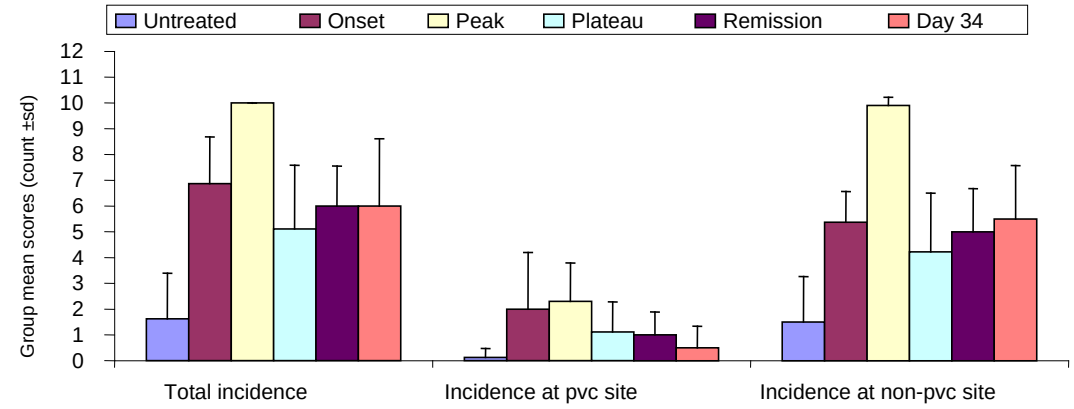

Figure 17a

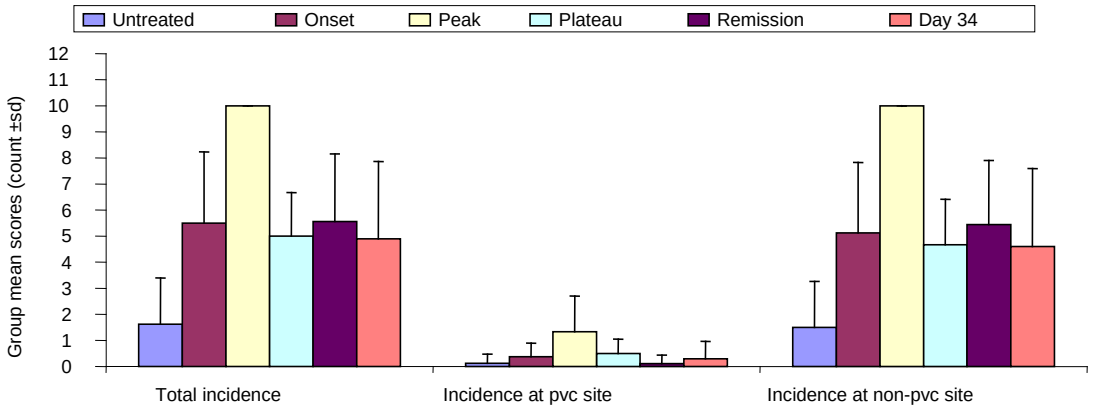

Figure 17b

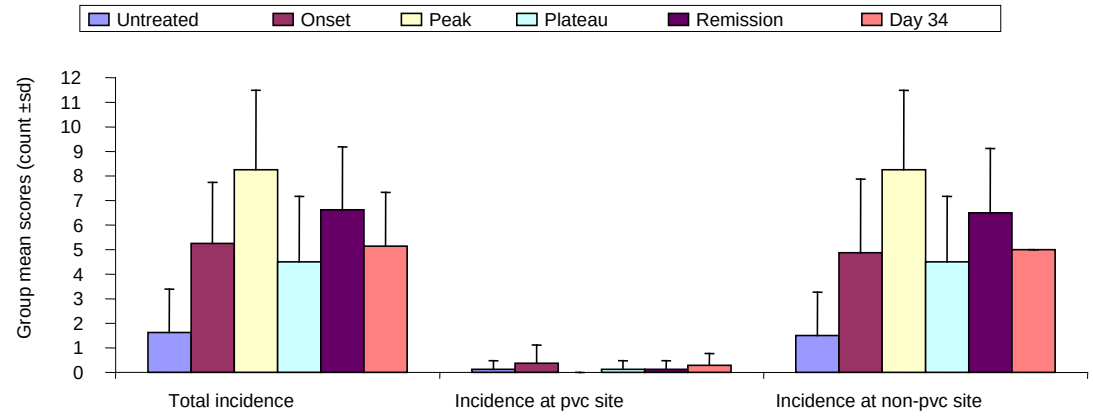

Figure 17c

Figure 17 Bar graphs showing the group mean C3 incidence at the time of sampling as determined by counting distinct areas of positive staining from ten areas selected randomly from two separate sections of spinal cord per rat (i.e. 20 separate areas counted / rat). (a) Successful EAE rats immunised with CNS material. (b) Unsuccessful rats immunised with CNS material. (c) Control (carbonyl iron) animals.

3.3.2 IgG staining

Figure 18 shows IgG immunostaining under light microscopy (x 40 objective). The animal from which the section below was taken was a female Lewis rat that had been immunised with CNS material and killed at peak EAE.

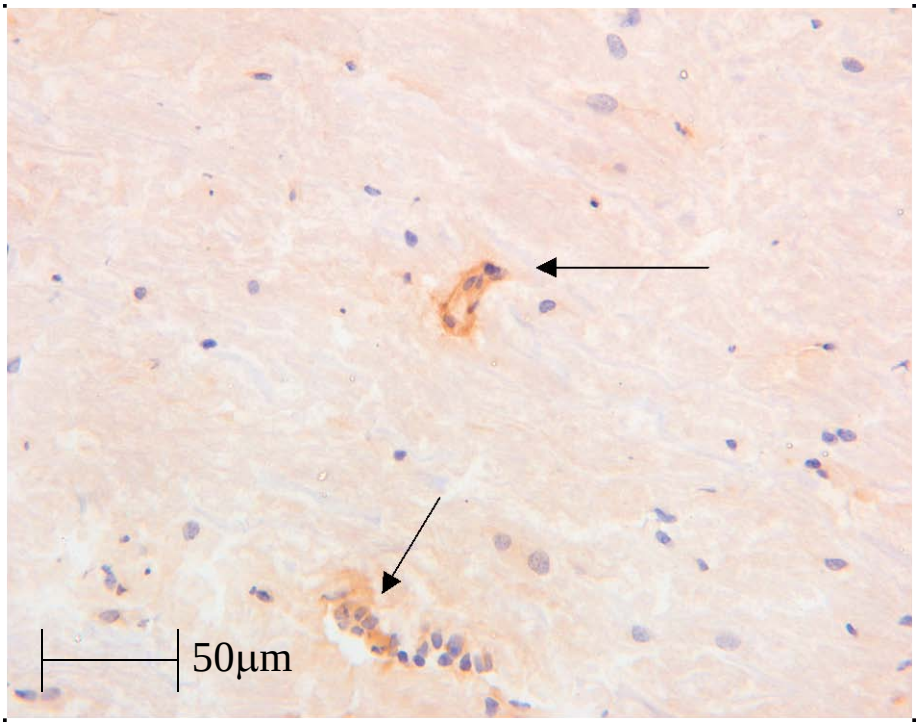

Figure 18 IgG staining using sheep anti-rat IgG:HRP and counter stained with haematoxylin, under light microscopy (x 40 objective). Arrows point to areas of positive IgG staining on a spinal cord section taken from a female Lewis rat that had been immunised with CNS material and killed at peak EAE.

Figures 19a-19c indicate that a relatively low incidence of IgG antibodies was present in the spinal cords of untreated animals.

In general, total specific IgG staining and staining at non-perivascular cuffing sites were comparable for animals showing signs of EAE, those failing to exhibit acceptable EAE signs and control animals sampled at plateau, remission and 'relapse' (Day 34). There was a marked increase in total IgG antibody incidence and the presence of IgG at non-perivascular sites in animals successfully developing EAE when sampled at onset and peak EAE, compared to IgG levels found at other sampling times and in unsuccessful and control animals. However, when unsuccessful and successful animals were compared to the carbonyl control groups using a Wilcoxon two sample test there was no statistically significant difference.

IgG detection at perivascular sites was negligible in the spinal cord of control animals, with a slightly increased incidence in animals that exhibited minimal (unsuccessful) EAE signs. Specific staining at perivascular sites in animals that successfully developed EAE signs had an increasing/decreasing trend in IgG antibody detection, with maximum IgG correlating with peak EAE. When unsuccessful animals were compared to the associated carbonyl control animals using a Wilcoxon two sample test there was no statistically significant difference. However when successful animals were compared to the associated carbonyl control animals using a Wilcoxon two sample test, the increase in IgG in the

spinal cord of animals sampled at peak EAE was found to be statistically significant ($P<0.05$).

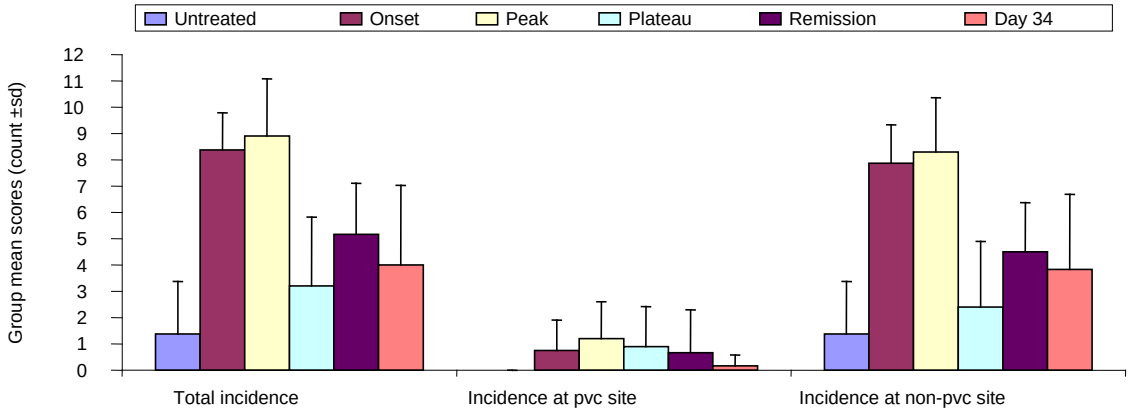

Figure 19a

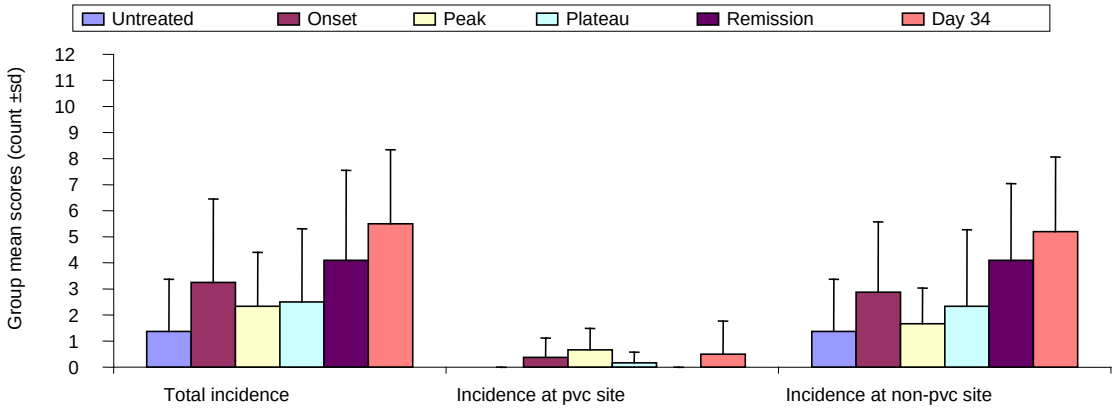

Figure 19b

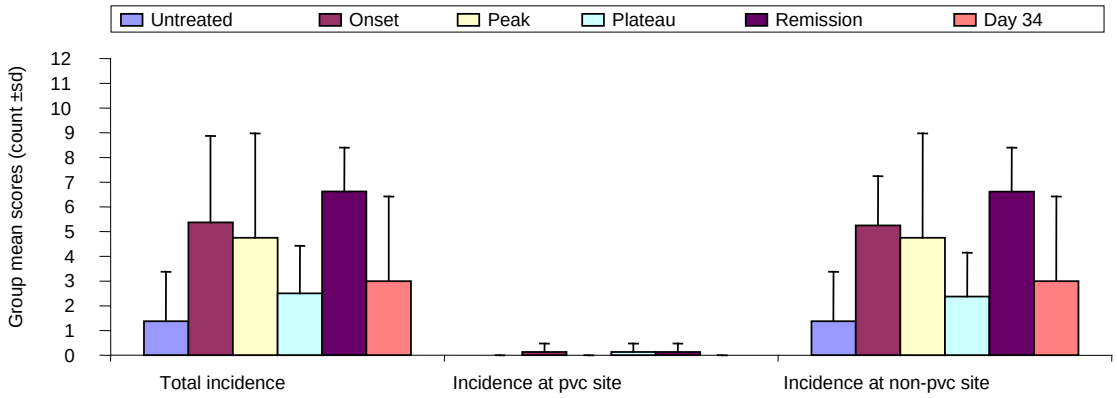

Figure 19c

Figure 19 Bar graphs showing the group mean IgG incidence at the time of sampling as determined by counting distinct areas of positive staining from ten areas selected randomly from two separate sections of spinal cord per rat (i.e. 20 separate areas counted / rat). (a) Successful EAE rats immunised with CNS material. (b) Unsuccessful rats immunised with CNS material. (c) Control (carbonyl iron) animals.

3.3.3 IgM staining

Figure 20 shows IgM immunostaining under light microscopy (x 40 objective). The animal from which the section below was taken was a female Lewis rat that had been immunised with CNS material and killed at the time of EAE onset.

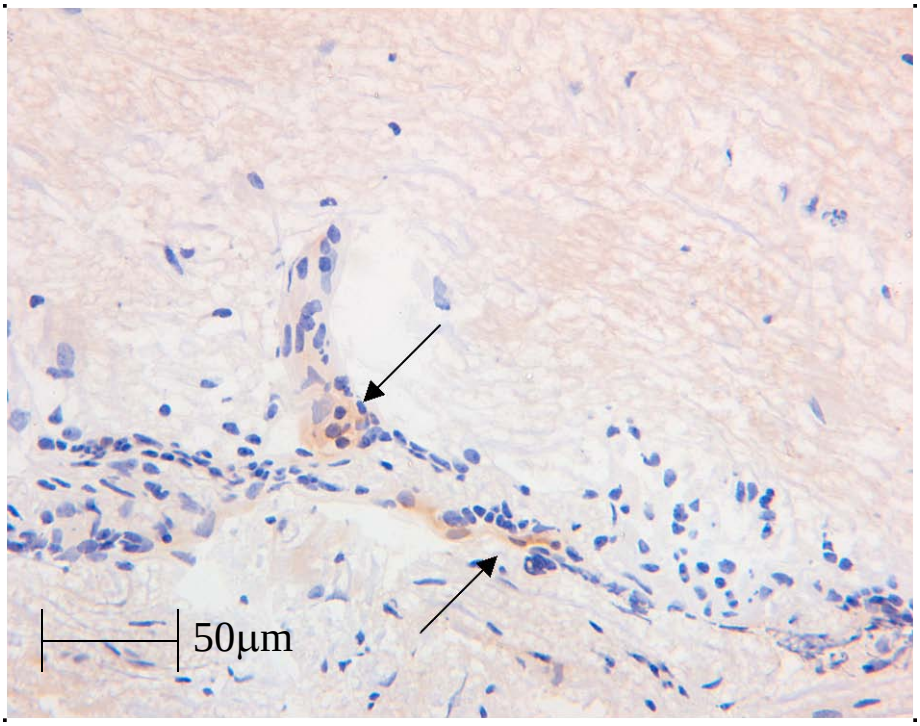

Figure 20 IgM staining using mouse anti-rat IgM:HRP and counter stained with haematoxylin, under light microscopy (x 40 objective). Arrows point to areas of positive IgM staining on a spinal cord section taken from a female Lewis rat that had been immunised with CNS material and killed at EAE onset.

Figures 21a-21c indicate that a negligible incidence of IgM antibodies was present in the spinal cords of untreated animals.

Total specific IgM staining and staining at non-perivascular cuffing sites was in general, notably higher in animals that successfully developed EAE compared to those unsuccessful in EAE development and control animals. With regards to total IgM incidence and detection at non-perivascular sites in successful animals, the peak occurred at onset sampling. When unsuccessful and successful animals were compared to the carbonyl control groups using a Wilcoxon two sample test there was no statistically significant difference.

IgM antibody detection at perivascular sites was negligible in the spinal cord of control animals and in those that failed to exhibit acceptable EAE signs. Specific staining at perivascular sites in animals successfully exhibiting EAE signs was negligible at onset, peak, remission and Day 34 (relapse) sampling points with peak IgM incidence occurring at plateau EAE. When unsuccessful and successful animals were compared to the carbonyl control groups using a Wilcoxon two sample test there was no statistically significant difference.

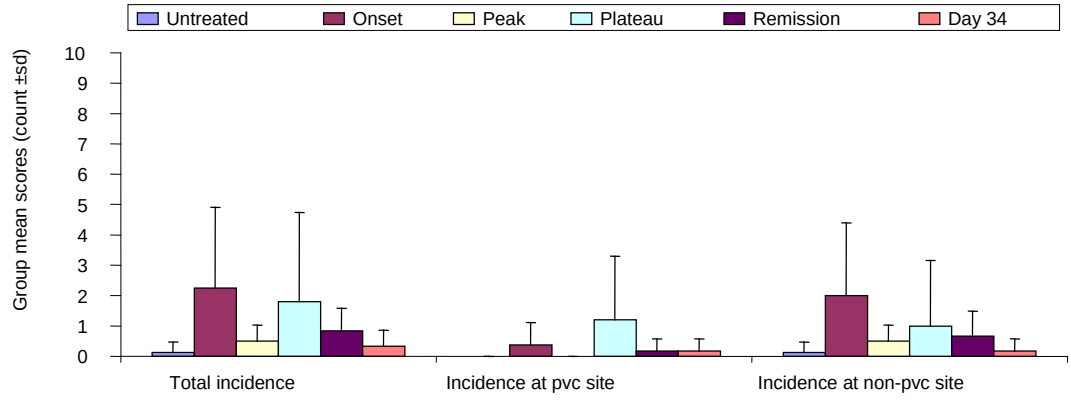

Figure 21a

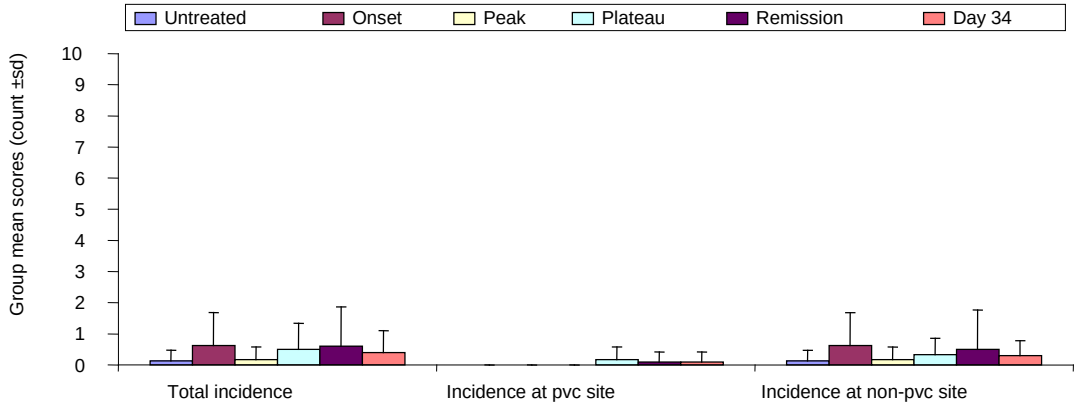

Figure 21b

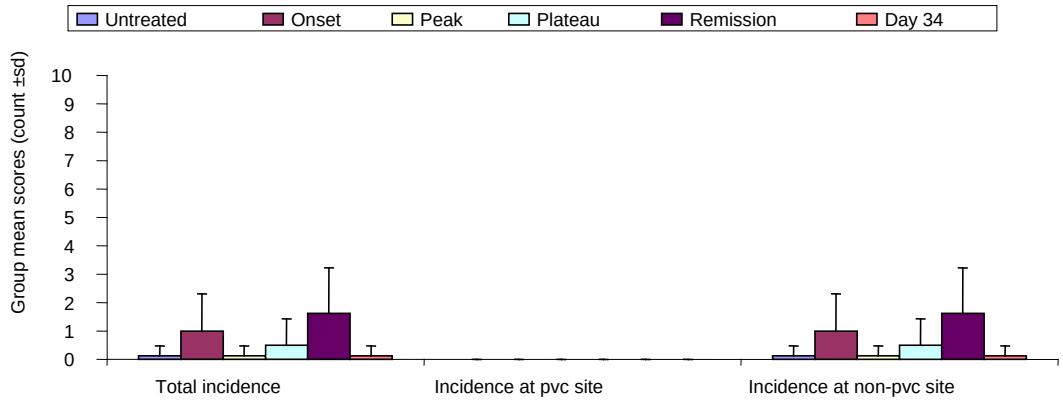

Figure 21c

Figure 21 Bar graphs showing the group mean IgM incidence at the time of sampling as determined by counting distinct areas of positive staining from ten areas selected randomly from two separate sections of spinal cord per rat (i.e. 20 separate areas counted / rat). (a) Successful EAE rats immunised with CNS material. (b) Unsuccessful rats immunised with CNS material. (c) Control (carbonyl iron) animals.

3.3.4 Perivascular cuffing

Figure 22 shows an area of perivascular cuffing in the spinal cord of a female Lewis rat immunised with CNS material under light microscopy (x 40 objective). This section was taken when EAE signs were at peak for this animal. Perivascular cuffing is seen as a dense area of cell infiltration around a vessel within the spinal cord.

Figure 23 shows a section of spinal cord from an untreated female Lewis rat under light microscopy (x 40 objective).

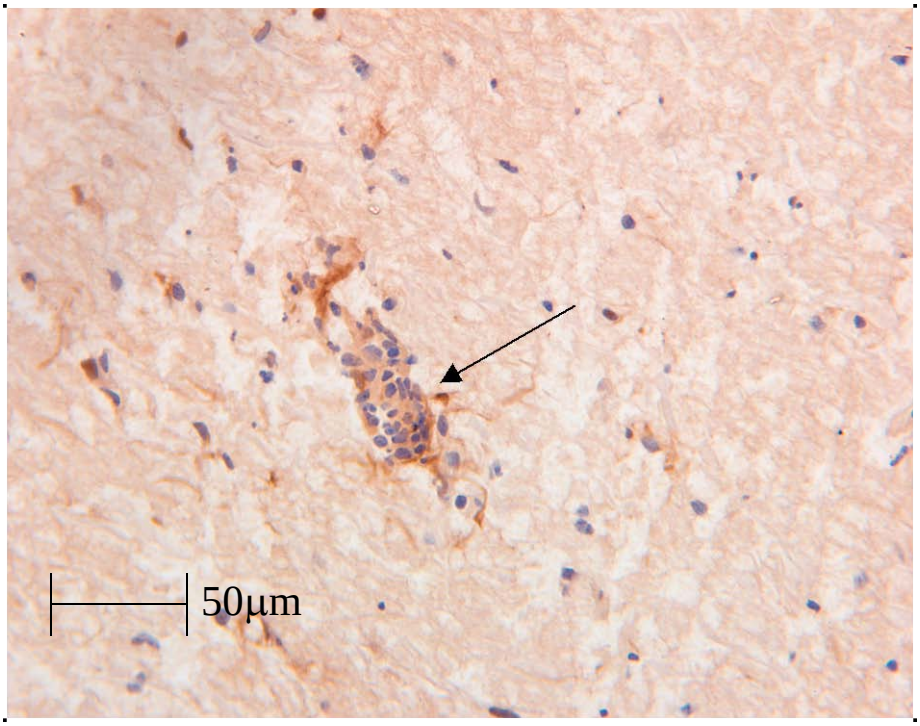

Figure 22 perivascular cuffing in the spinal cord of a female Lewis rat immunised with CNS material under light microscopy (x 40 objective). This section was stained for C3 using sheep anti-rat complement C3 and counter stained with haematoxylin. This section has been taken when EAE signs were at peak for this animal. The arrow points to an areas of perivascular cuffing as seen as a dense area of cell infiltration around a vessel within the spinal cord.

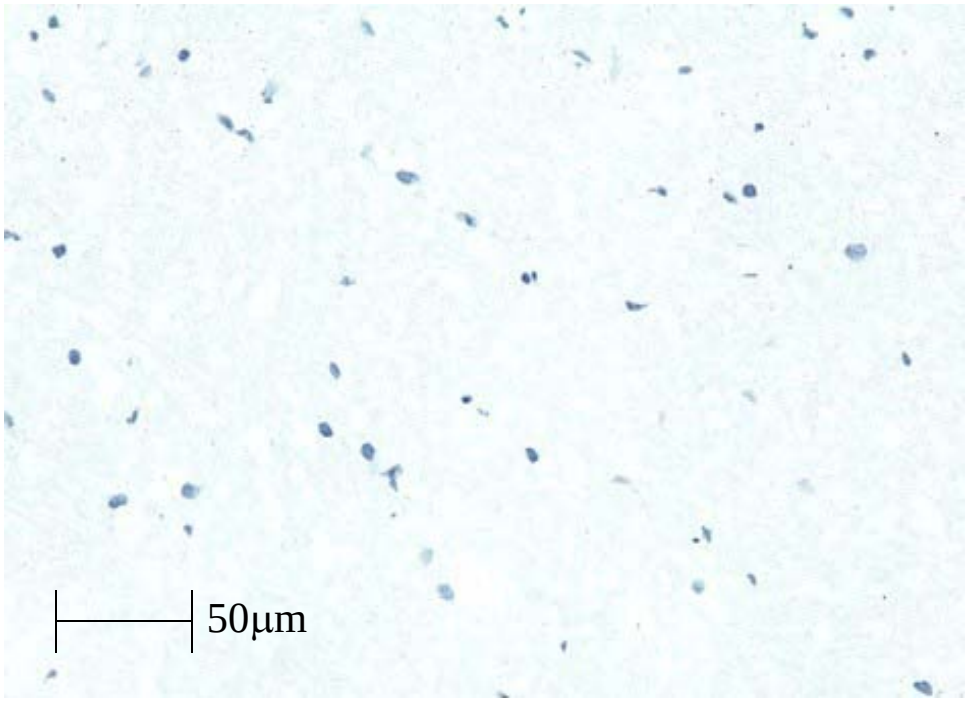

Figure 23 A section of spinal cord from an untreated female Lewis rat under light microscopy (x 40 objective) counter stained with haematoxylin.

Figure 24 shows how the incidence of perivascular cuffing in the spinal cord of animals immunised with CNS material (with carbonyl iron) increases from onset of EAE signs, reaches maximal cuffing when EAE signs are at a plateau and then diminishes thereafter. This trend can be clearly seen in both EAE successful and unsuccessful animals alike. In general, the incidence of perivascular cuffing in the spinal cord of control animals remains comparable at all sample time-points.

When compared using a Wilcoxon two sample test, the increased incidence of perivascular cuffing in EAE successful animals at onset, peak, plateau, remission and Day 34 was found to be statistically significant ($P<0.01$ (onset), $P<0.001$ (peak), $P<0.05$ (plateau), $P<0.001$ (remission) and $P<0.05$ (Day 34)) when compared to the control animals. This was in contrast to EAE unsuccessful animals for which there was a statistically significant increase in perivascular cuffing when compared to the control animals at peak and plateau only ($P<0.001$ and $P<0.01$, respectively).

3.3.5 Negative control group

Comparisons were made to animals treated with carbonyl iron alone. Animals in this control group were treated exactly the same as the EAE animals with the exception that they did not receive CNS material along side the adjuvant (carbonyl iron). The purpose of this group was to see if carbonyl iron alone had the potential to induce an EAE state and cause an inflammatory response within the CNS. By comparing the EAE animals to this group any potential adjuvant effect was eliminated. A small number of

untreated animals were included for reference purposes only and were not used for comparison.

Untreated animals showed no clinical signs, negligible perivascular cuffing, no sign of IgM and minimal IgG, C3, B cell staining when compared to the carbonyl iron group and EAE positive animals.

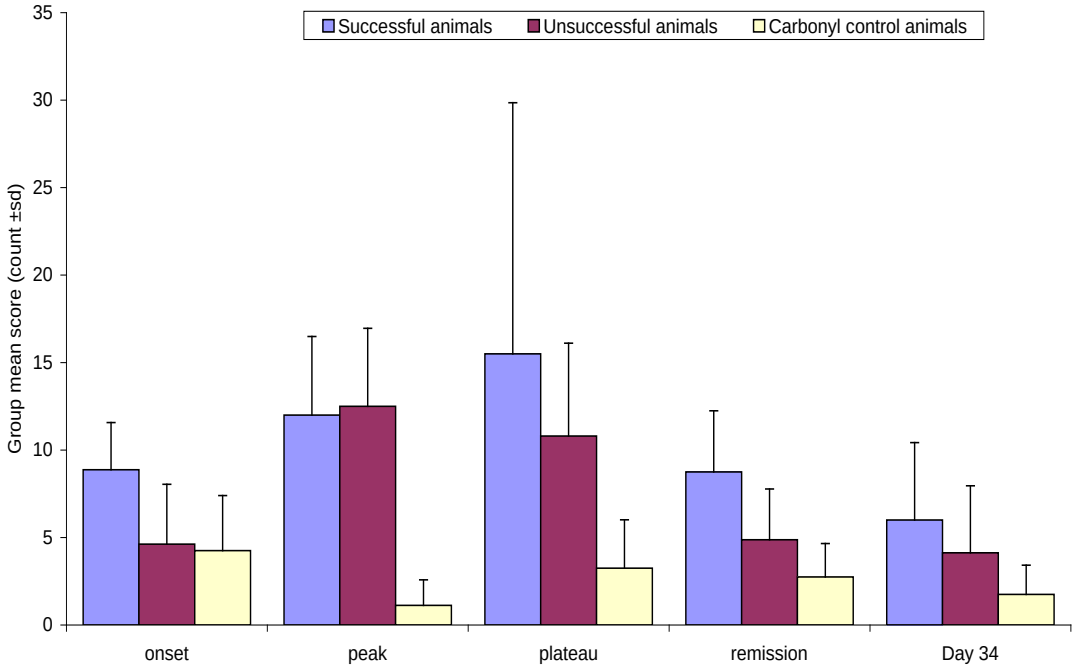

Figure 24 Bar graphs showing the group mean perivascular cuffing incidence at the time of sampling as determined by counting distinct areas of the accumulation of inflammatory deposits from ten areas selected randomly from two separate sections of spinal cord per rat (i.e. 20 separate areas counted / rat) for successful EAE rats immunised with CNS material, unsuccessful rats immunised with CNS material and control (carbonyl iron) animals. The individual counts from successful animals sampled at plateau ranged from 1 to 36 and as such this mean is associated with a large standard deviation.

3.3.6 B cell staining

Figure 25 shows B cell immunostaining under light microscopy (x 40 objective). The animal from which the section below was taken was a female Lewis rat that had been immunised with CNS material and killed at the time of EAE onset.

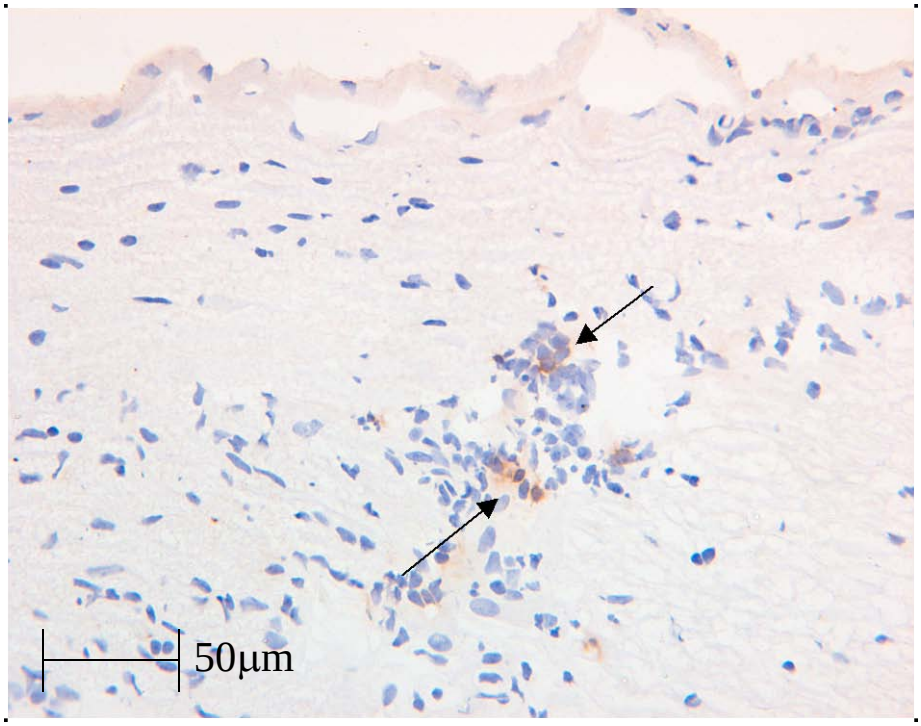

Figure 25 B cell staining using mouse anti-rat B cells and counter stained with haematoxylin, under light microscopy (x 40 objective). Arrows point to areas of positive B cell staining on a spinal cord section taken from a female Lewis rat that had been immunised with CNS material and killed at EAE onset.

Figures 26a-26c indicate that B cells were not detected in the spinal cords of untreated animals.

In general, total specific B cell staining was comparable between successful and unsuccessful rats sampled at peak, plateau, remission and Day 34 (relapse). B cell incidence in animals successfully developing EAE sampled at onset was notably higher than B cell detection in unsuccessful animals at this time-point. The highest total B cell incidence and presence at non-perivascular sites in control animals was observed at onset and was comparable to that seen in unsuccessful animals at this time-point. Minimal B cell staining was observed at all other time-points in the control group.

When unsuccessful and successful animals were compared to the associated carbonyl control animals using a Wilcoxon two sample test there was no statistically significant difference.

B cell detection at perivascular sites was negligible in the spinal cord of control animals. A similar incidence was apparent at perivascular cuffing sites for successful and unsuccessful animals sampled at onset, plateau, remission and Day 34. Peak B cell staining correlated with peak EAE activity, with a considerably higher detection level in animals successfully developing EAE. When unsuccessful and successful animals were compared to the associated carbonyl control animals using a Wilcoxon two sample test, the increase in B cells in the spinal cord of animals sampled

at peak EAE was found to be statistically significant ($P<0.05$ and $P<0.001$, respectively).

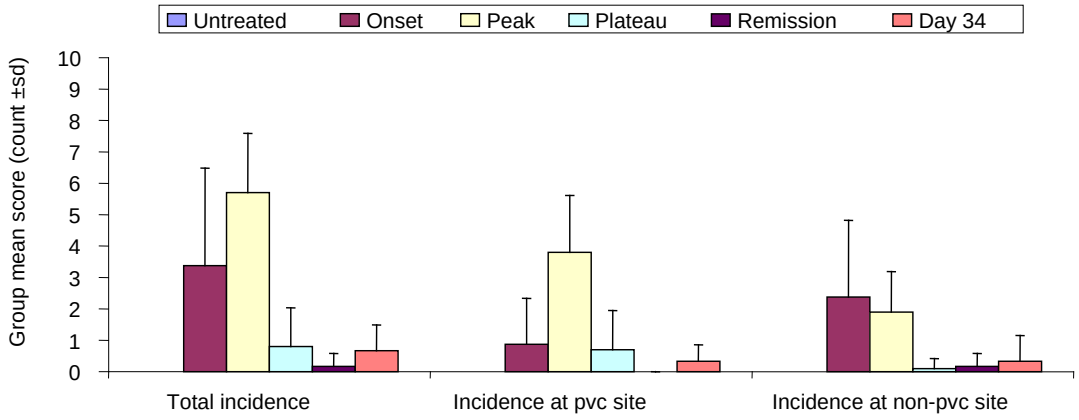

Figure 26a

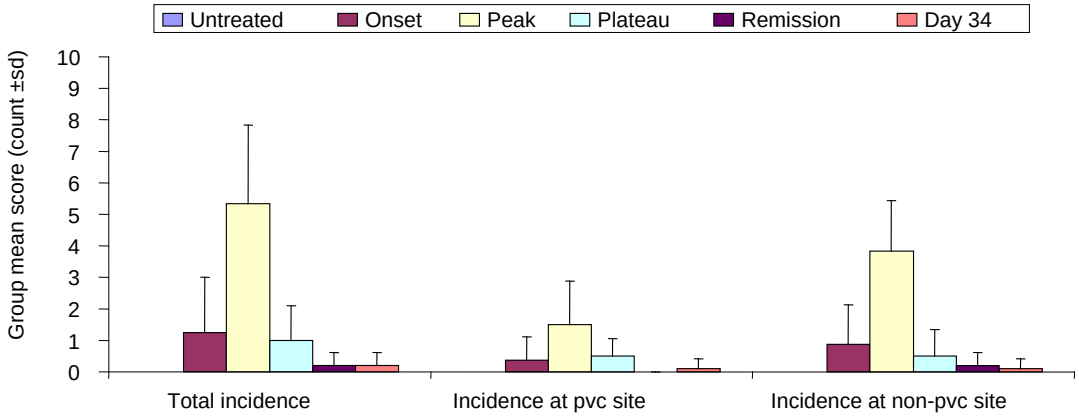

Figure 26b

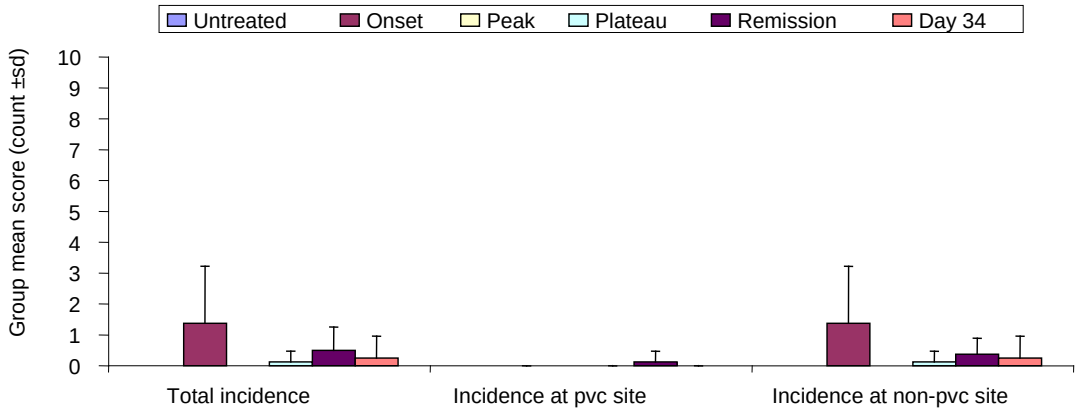

Figure 26c

Figure 26 Bar graphs showing the group mean B cell incidence at the time of sampling as determined by counting distinct areas of positive staining from ten areas selected randomly from two separate sections of spinal cord per rat (i.e. 20 separate areas counted / rat). (a) Successful EAE rats immunised with CNS material. (b) Unsuccessful rats immunised with CNS material. (c) Control (carbonyl iron) animals.

3.3.7 DAPI staining

Preliminary investigations into the feasibility of using DAPI labelled DNA to probe for the presence of anti-ssDNA and anti-dsDNA antibodies in *situ* were encouraging. Sections of spinal cord from all successful EAE rats sampled at peak and plateau EAE (10 animals in total) in addition to untreated (4 animals) and associated carbonyl iron control animals (8 animals) were fixed in acetone, which in general showed better morphology than sections air-dried without fixation. Staining was not apparent when the probes were applied to the spinal cord of untreated rats. When applied to EAE rat spinal cord sections, the sections to which the ssDNA probe was added showed slight fluorescence, whereas the addition of the dsDNA probe produced slightly better results with regards to staining.

Three main types of staining were observed following incubation with the DAPI/dsDNA probe; small discrete, circular areas of fluorescence (e.g. figure 34), fluorescence around the perimeter of a largish circular 'cell', with fluorescence gradually diminishing with increasing distance from the cell body (e.g. figure 27) and fluorescence around the perimeter of a cell (e.g. figure 30).

These areas of positive staining were investigated in a number of ways. Fluorescence was observed under light in the green wavelength (510 nm) and compared to fluorescence under light in the red wavelength (650 nm) (figures 32-35). This was purely done as a matter of interest rather than

being based on a scientific rationale. In general there was little or no difference in fluorescence, with similar intensity and staining patterns observed under both wavelengths. In addition to red and green wavelength light DAPI/dsDNA staining was initially observed under ultraviolet light, this being the wavelength recommended following DAPI staining for nuclei DNA (figures 36 and 37). Under ultraviolet light, the intensity of the fluorescence seen as excess DAPI bound to nuclear DNA obscured any potential positive staining for anti-dsDNA antibodies. Dual DAPI and H & E staining was also employed to further investigate areas of fluorescence.

Although the area of staining seen in figure 27 was considered to be too large for any cell and as such is most likely an artefact of slide preparation, similar staining patterns were observed in a number of sections which were not considered to be artefacts. Figure 28 shows a group of cellular structures for which fluorescence is radiating from the outer surfaces, such staining patterns show a similarity to that of Russell body cells (figure 29) [reference unavailable]. Russell bodies are small spherical intracytoplasmic hyaline bodies, which are common in plasma cells and are frequently seen in cases of chronic inflammation. As inflammatory is a major occurrence in EAE this finding is certainly a possibility.

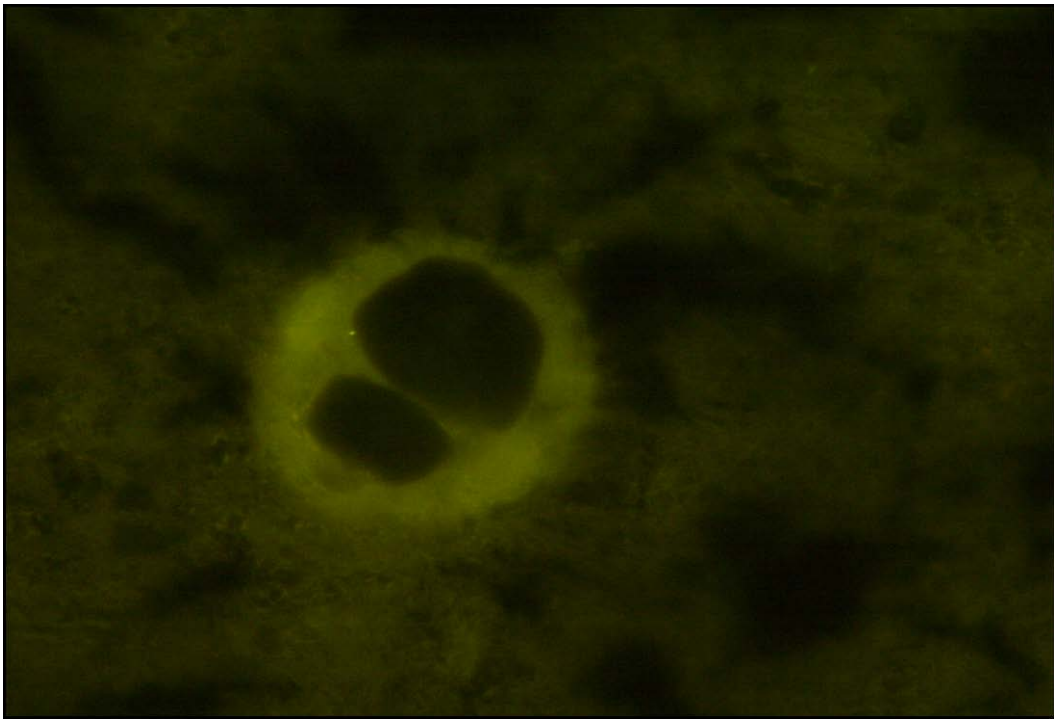

Figure 27 DAPI/dsDNA probe staining (x 100 objective). The above shows DAPI/dsDNA fluorescence on a spinal cord section taken from a female Lewis rat that had been immunised with CNS material and killed at peak EAE.

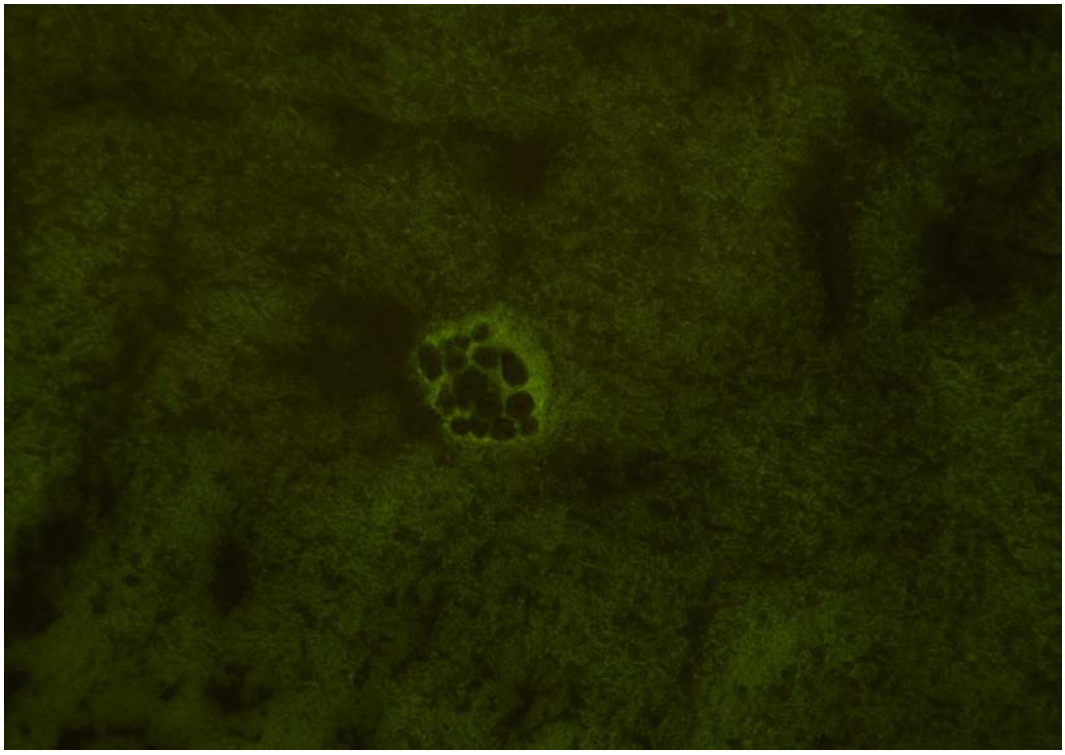

Figure 28 DAPI/dsDNA probe staining (x 100 objective). The above shows DAPI/dsDNA fluorescence on a spinal cord section taken from a female Lewis rat that had been immunised with CNS material and killed at peak EAE.

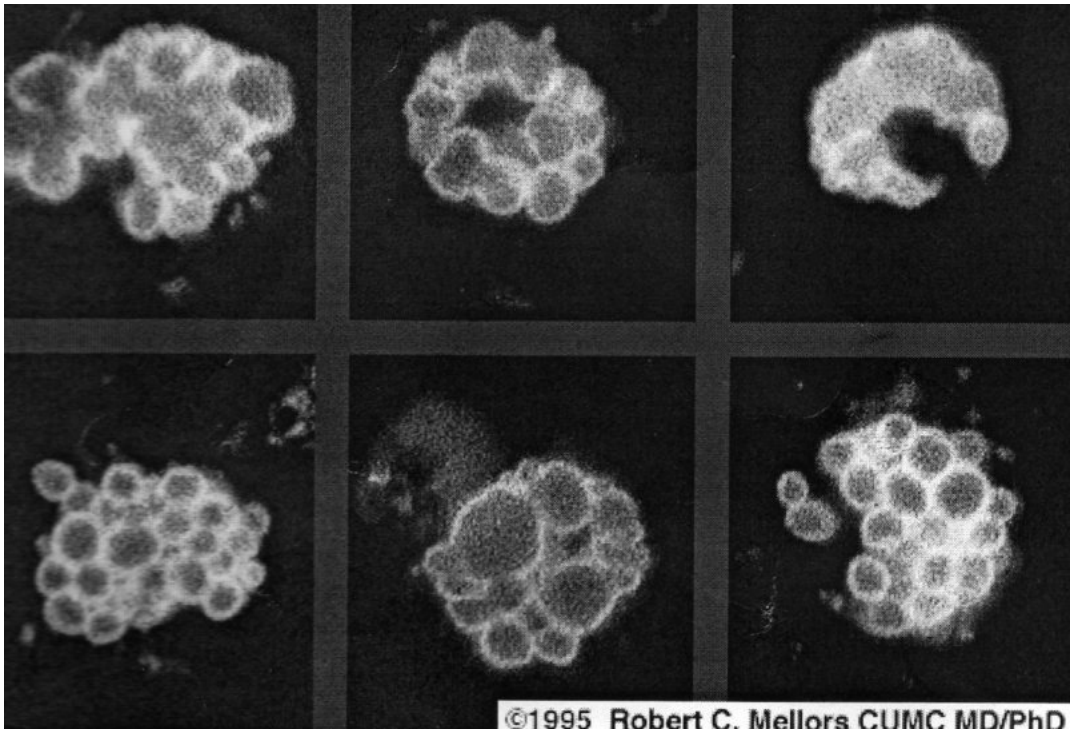

Figure 29 Plasma cells forming Russell bodies.

Figures 30 - 33 show positive DAPI/dsDNA staining round the surface of B cells in rat (untreated) spleen sections as indicated by the arrows, which is suggestive of secretion of anti-dsDNA antibodies. In normal animals it is not unusual to find low levels of anti-dsDNA antibodies. These natural antibodies tend to be IgM and are non-pathogenic [Datta (1983)].

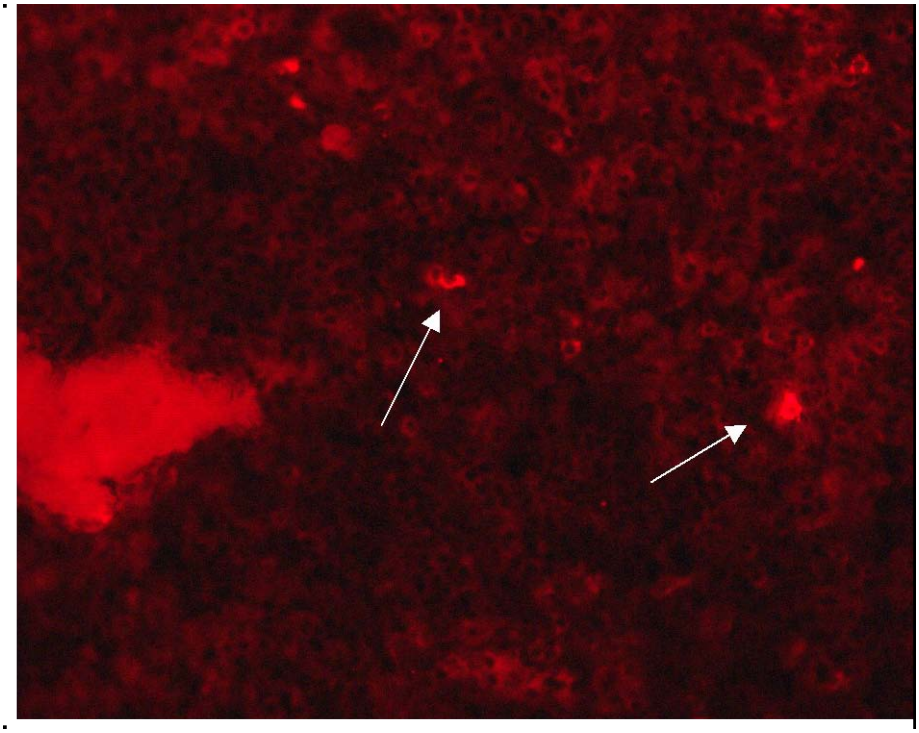

Figure 30 DAPI/dsDNA probe staining as visualised under 650 nm wavelength of light, of a spleen section taken from an untreated rat (x 40 objective). The white arrows point to positive staining around the surface of B cells.

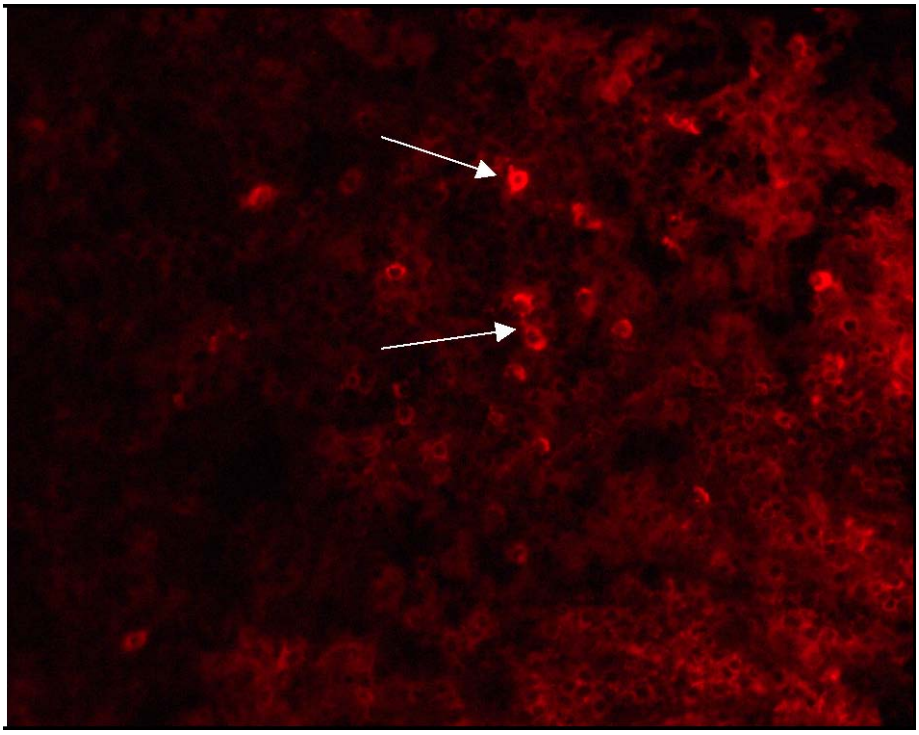

Figure 31 DAPI/dsDNA probe staining as visualised under under 650 nm wavelength of light, of a spleen section taken from an untreated rat (x 40 objective). The white arrows point to positive staining around the surface of B cells.

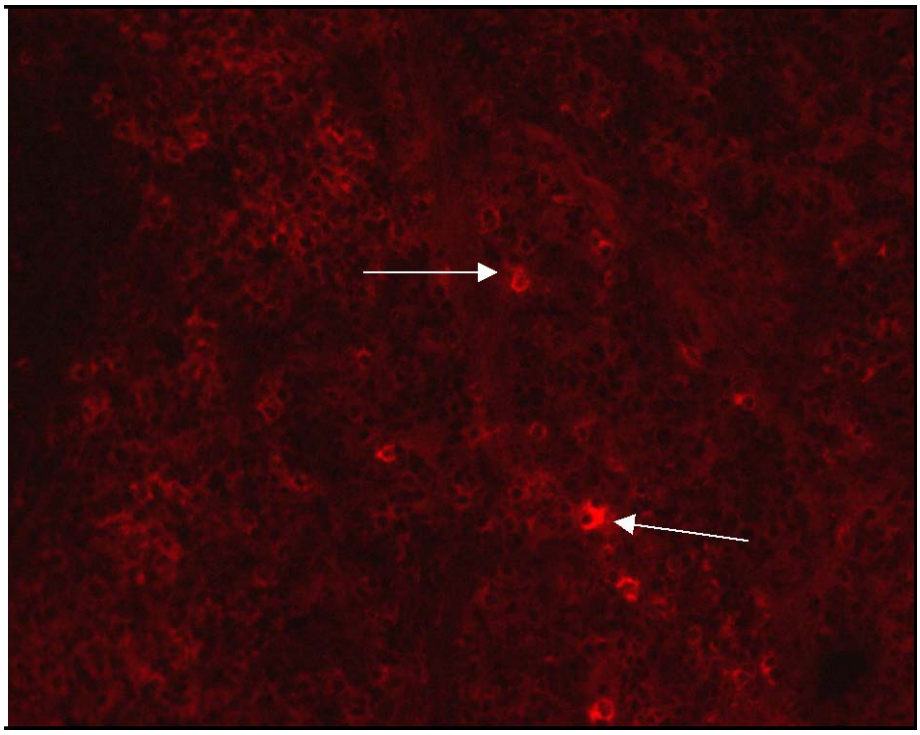

Figure 32 DAPI/dsDNA probe staining as visualised under 650 nm wavelength of light, of a spleen section taken from an untreated rat (x 40 objective). The white arrows point to positive staining around the surface of B cells.

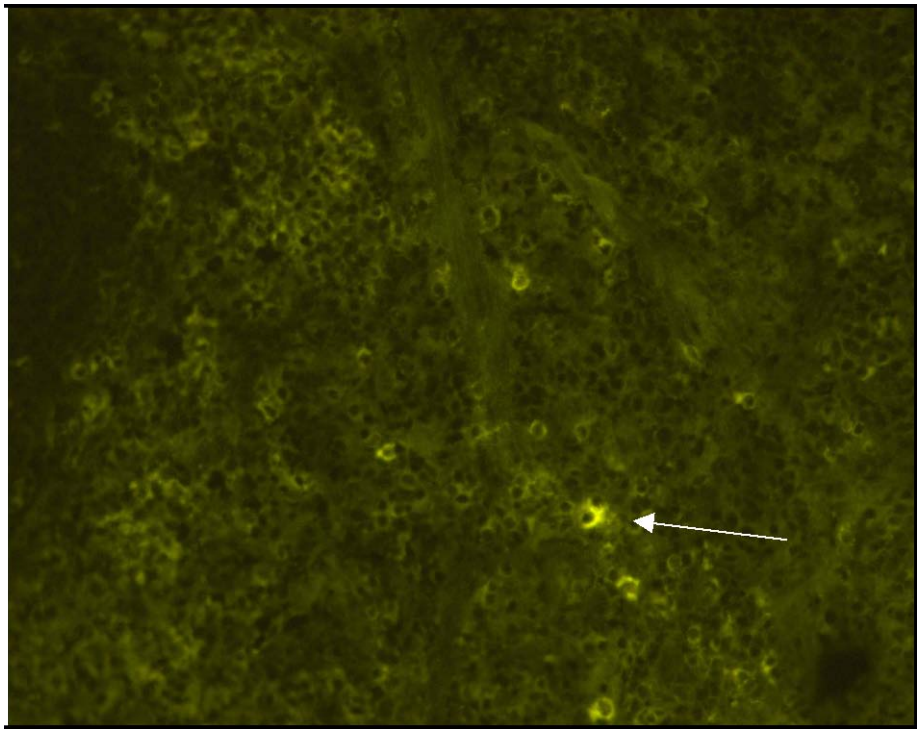

Figure 33 DAPI/dsDNA probe staining as visualised under 510 nm wavelength of light, of a spleen section taken from an untreated rat (x 40 objective). The white arrow points to positive staining around the surface of B cells.

A similar pattern of staining can be seen in figures 34 and 35 which would suggest the presence of anti-dsDNA antibodies in this section of spinal cord taken from a rat displaying signs of peak EAE.

In addition, the arrangement of the staining (indicated by the arrow) seen in this section is such that it appears to be on the outer edge of a structure, which could possible be a lesion. This is typical of anti-dsDNA antibodies in MS [Williamson *et al*. (2001)].

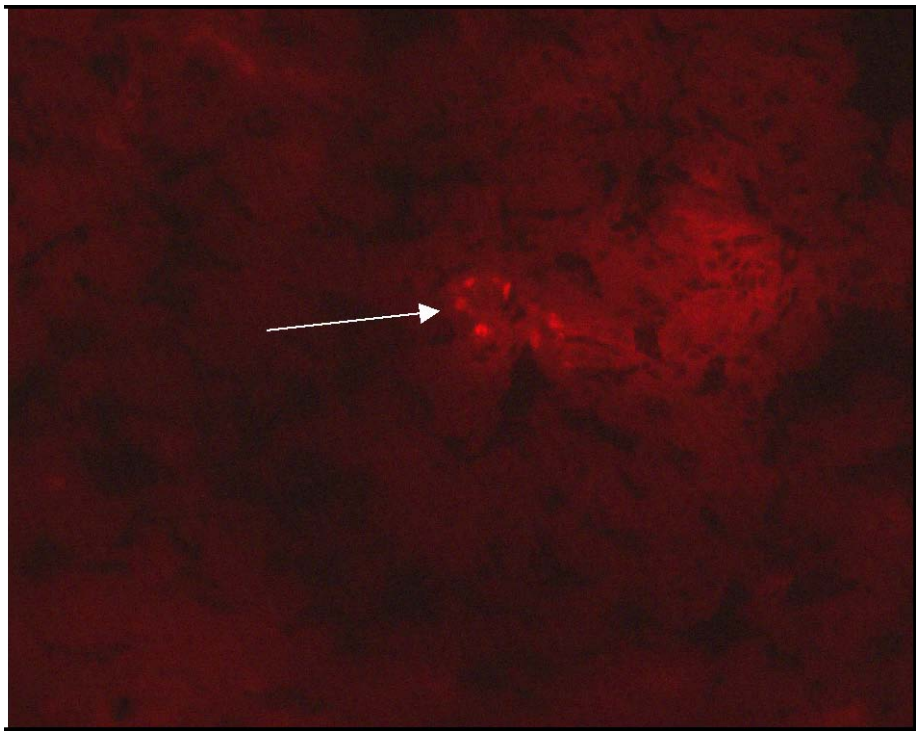

Figure 34 DAPI/dsDNA probe staining as visualised under 650 nm wavelength of light, of a spinal cord section taken from a rat showing peak EAE (x40 objective). The arrow points to positive staining around an oval structure, possibly a lesion.

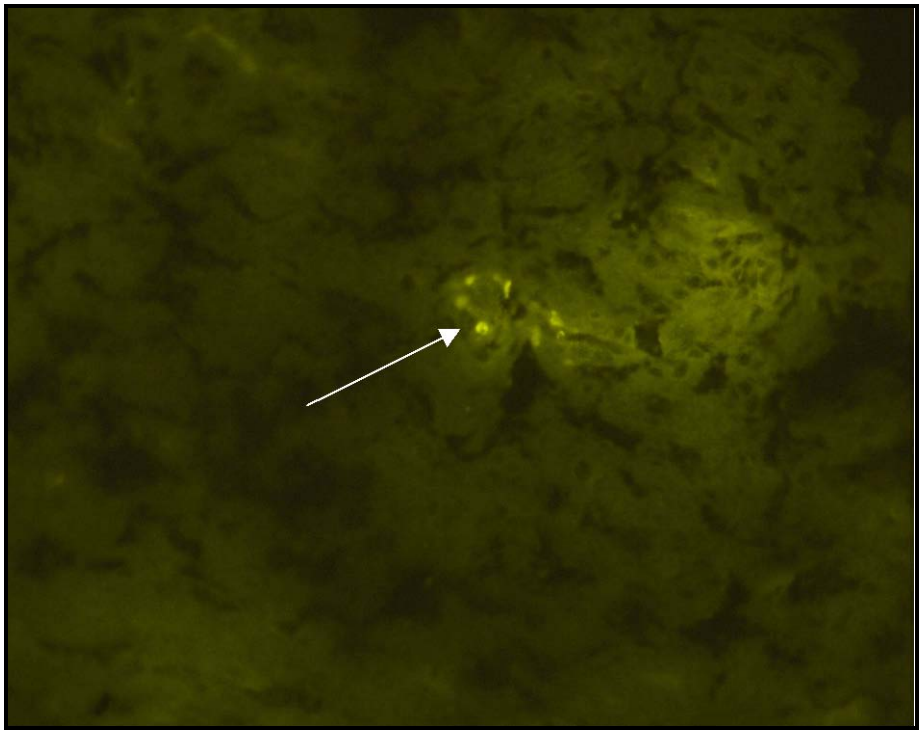

Figure 35 DAPI/dsDNA probe as visualised under 510 nm wavelength of light, of a spinal cord section taken from a rat showing peak EAE (x 40 objective). The arrow points to positive staining around an oval structure, possibly a lesion.

Figures 36 and 37 show DAPI/dsDNA probe staining of a section of spinal cord taken from a rat showing peak EAE signs. The nuclear DNA can be seen in the centre of the blue stained cells as a pale blue fluorescent area. Figure 37 shows there to be an area of interest (indicated by the arrow) to the right of the section which has the appearance of a secretory B cell with reddish staining diffusing from its surface. To the left of the section there is another very small area of red staining (indicated by the arrow) because of its small size any distinct pattern is prevented from being determined.

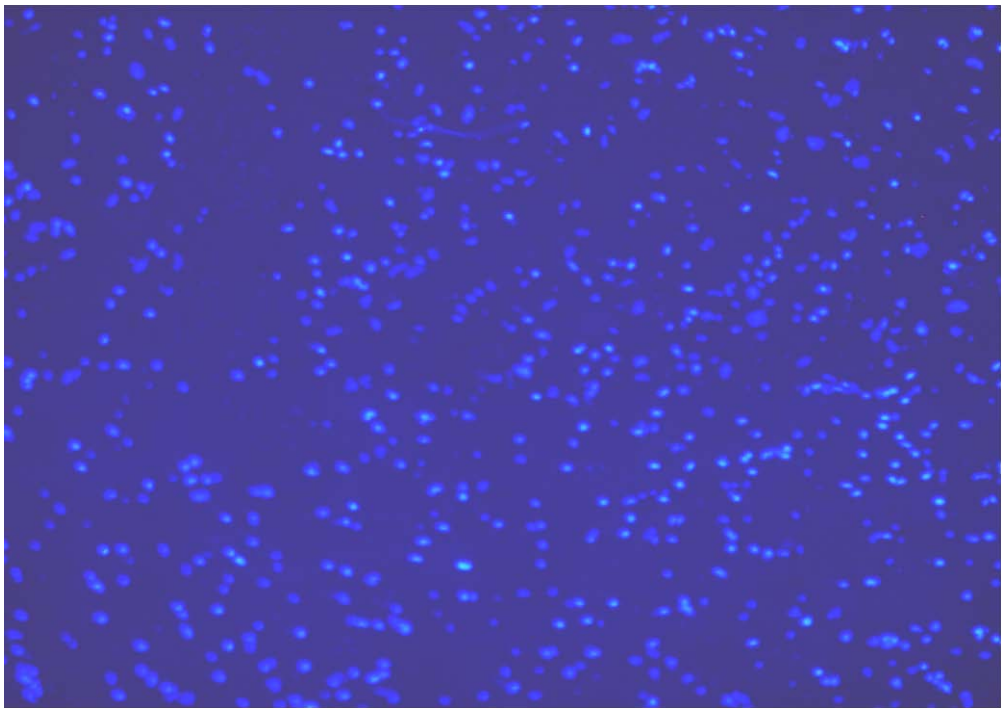

Figure 36 DAPI/dsDNA staining of cell nuclei as visualised under UV light of a spinal cord section taken from a rat showing peak EAE (x 40 objective).

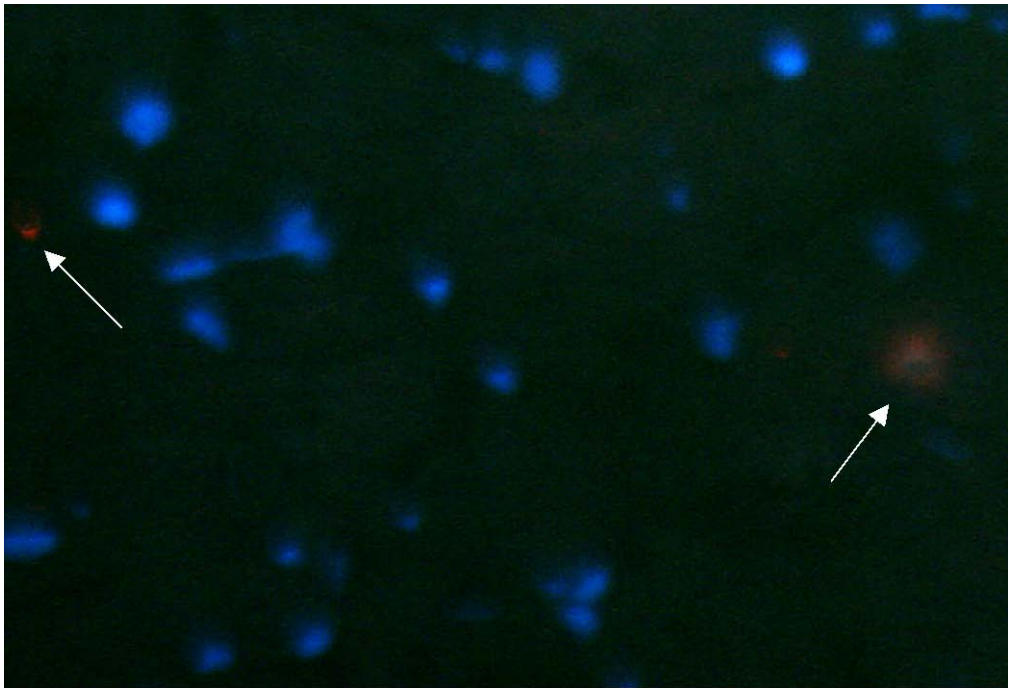

Figure 37 DAPI/dsDNA staining of cell nuclei as visualised under UV light of a spinal cord section taken from a rat showing peak EAE, unknown magnification.

3.3.8 Anti-dsDNA antibody determination in the rats using Alexa Fluor/dsDNA probe

Alexa Fluor/dsDNA probe on EAE rat brain sections was investigated. The apparent lack of fluorescence in human spinal cord and the successful detection in human brain sections suggested it would be a worthwhile exercise to examine the brains of rats killed at peak EAE. In support of this, in MS patients, the lesions are more often found in the brain than in the spinal cord [MedHelp. Multiple Sclerosis Community].

No positive staining was found in any of the sections of rat brain examined. This may have been due to the sections taken not containing any active lesions. Human brain sections examined in this project had been screened and selected specifically due to the presence of active lesions. The sections taken from animals were randomly selected without any indication of a lesion being present.

3.4 Clinical signs of EAE – *in vivo* phase – Biozzi (ABH) mouse (remitting/relapsing)

Due to the limitations of the acute EAE model in the rat, in that the antibody response to subsequent disease flares was not possible to assess, attempts were made at developing a reliable relapsing-remitting EAE model in the mouse. It was anticipated that with each EAE episode a greater antibody response would be produced, resulting in a higher incidence of anti-dsDNA antibodies in the CNS.

There were in total eight attempts of getting the Biozzi (ABH) mouse EAE model to work.

Part I: This method was based on published information [Liblau *et al*. (1997) and Heremans *et al.* (1999)] and modified based on previous experience with other immunological animal models, i.e. the collagen type II arthritis in the rat.

Group housed female Biozzi (ABH) mice (approximately 6 weeks old) were injected with finely chopped Biozzi (ABH) mouse spinal cord in Freund's adjuvant (FA). The route of administration was intradermal into the base of the tail. EAE signs were not detected in any animal.

Part II: The lack of EAE signs in Part I, was thought to be due to poor preparation of the spinal cord. For this phase of the validation, spinal cord

was homogenised, rather than finely chopped, to form a uniform and fine suspension.

Group housed female Biozzi (ABH) mice (approximately 6 weeks old) were injected with homogenised Biozzi (ABH) mouse spinal cord in Freund's adjuvant. The route of administration was intradermal into the base of the tail. EAE signs were seen in two out of eight mice with one of these animals displaying a completely limp tail. Weight loss was also apparent for these mice at the time of the appearance of EAE signs. Signs were first apparent on Day 18 and had disappeared by Day 26. Neither animal relapsed. Another mouse displayed a transient weight loss, but showed no conclusive sign of EAE.

Part III: Due to limited success in Part II of the validation, it was considered necessary to change the EAE induction protocol. Three different methods of induction were examined. Spinal cord homogenised with carbonyl iron dosed intraperitoneally was assessed, this being the method of induction in the rat EAE model which was found to be successful. The use of bovine and murine myelin basic protein has been documented in published literature [Asensio *et al*. (1999)] and as such were also considered worthy of investigation.

Group housed female Biozzi (ABH) mice (approximately 6 weeks old) were given an intraperitoneal injection of homogenised Biozzi (ABH)

mouse spinal cord in carbonyl iron (100 mg/mL (1:1)) or an intradermal injection of bovine or murine MBP in FA at the base of the tail.

EAE signs were seen in two out of six mice treated with murine MBP. Signs were first apparent on Day 20 and by Day 27 had completely disappeared. Both animals exhibited a completely limp tail, neither showed any sign of relapse.

EAE was not exhibited by any of the mice treated with bovine MBP or Biozzi (ABH) spinal cord in carbonyl iron.

Part IV: Due to limited success it was considered appropriate to return to the original induction protocol. However, in this phase of the study the animals were weighed daily to see if a cyclic decrease in bodyweight was present, even in the absence of EAE specific signs.

Group housed female Biozzi (ABH) mice (approximately 5 weeks old) were injected with homogenised Biozzi (ABH) mouse spinal cord in Freund's adjuvant. The route of administration was intradermal into the base of the tail. The animals were weighed daily to closely monitor bodyweights. EAE signs (a completely limp tail) were seen in one out of ten mice. Signs were first apparent on Day 21 and had gone by Day 24. There was no indication of a relapse.

A noteworthy observation was made with regards to all mice receiving the encephalitogenic agent, this being that from Day 78 all mice had hair loss around the whisker area. There was no indication that scratching or excess grooming had taken place. Although not related to this project, this finding is of some interest in that Alopecia Areata is caused by an autoimmune response. Why this should occur following this particular immunisation protocol is not known.

Part V: A possible cause of the lack of anticipated response in the previous validation attempts was thought possibly to be due to the encephalitogenic agent being too similar to 'self', i.e. Biozzi (ABH) spinal cord. For the acute EAE study, rats are injected with guinea-pig spinal cord and successfully develop EAE signs. In order to investigate this further, for this phase of the study CD-1 mouse and guinea-pig spinal cord were used.

Group housed female Biozzi (ABH) mice (approximately 6 weeks old) were injected with homogenised CD-1 mouse or guinea pig spinal cord with Freund's adjuvant intradermally into the base of the tail. A slightly limp tail was seen for one out of five mice dosed with CD-1 mouse spinal cord. Signs were first apparent on Day 17 and had disappeared by Day 21. There was no sign of relapse.

EAE was not seen in mice treated with guinea-pig spinal cord.

Part VI: Following advice from a contact at St Bartholomew's Hospital Medical College, London, validation was attempted using male Biozzi (ABH) mice. Although female mice are in general more susceptible to EAE, success of inducing EAE in females can be varied due to fluctuating hormone levels. The mice were housed individually to prevent in-house fighting, thus minimising the potential to increase stress hormone levels such as corticosteroids, which have the affect of inhibiting the required autoimmune response. The animals used were considerably older than previously tested and were considered to be an optimum age for EAE induction.

Individually housed male Biozzi (ABH) mice (approximately 10 weeks old) were injected with homogenised CD-1 mouse spinal cord and Freund's adjuvant intradermally into the base of the tail. EAE signs were not detected in any animal.

Part VII: Due to a failure of EAE induction, further advice was received which indicated that freeze dried spinal cord was preferable to homogenised material and also Freund's adjuvant should be supplemented with *mycobacterium*. Subcutaneous administration into each flank was suggested [Liblau *et al*. (1997) and Heremans *et al.* (1999)].

Individually housed male Biozzi (ABH) mice (approximately 12 weeks old) were injected with freeze dried CD-1 mouse spinal cord, Freund's adjuvant

and M*ycobacterium butyricum* (heat killed) subcutaneously into the flank. EAE signs were not detected in any animal.

Part VIII: A lack of EAE signs in mice in Part VII led to a final attempt at inducing EAE in the Biozzi (ABH) mouse. For this phase intradermal injection into the base of the tail was used as before, but this time *Mycobacteria* were added to the Freund's adjuvant.

Individually housed male Biozzi (ABH) mice (approximately 10 weeks old) were injected with freeze dried CD-1 mouse spinal cord, Freund's adjuvant and *Mycobacterium butyricum* (heat killed) intradermally into the base of the tail. EAE signs were not detected in any animal.

3.5 Anti-dsDNA antibody determination in the mouse using Alexa Fluor/dsDNA probe

Examination of Biozzi (ABH) mouse brains and spinal cords using Alexa Fluor/dsDNA probe produced no positive results (figure 38).

Alexa Fluor/dsDNA probe staining of mouse CNS sections in general produced similar results to those seen with DAPI/dsDNA probe staining. Areas of fluorescence were seen and ranged from small discrete areas (figure 47) to larger B cell type structures similar to those seen in figure 42. Fluorescence was apparent in both EAE-treated and untreated mouse spinal cords alike.

Areas of fluorescence were extensively investigated using dual staining methods (Alexa Fluor/dsDNA probe and H & E). Dual staining in many cases showed there to be little correlation of fluorescent areas with cellular structures and as such was thought to be due to autofluorescence of the tissue or an artefact introduced during the preparation of the sections. For example, the large fluorescence structure seen in figure 39 (indicated by the arrow), although initially promising, once stained with H & E (figures 40 and 41) appeared to be out of the plane of section, suggesting that this finding is most likely to be an artefact of the staining process.

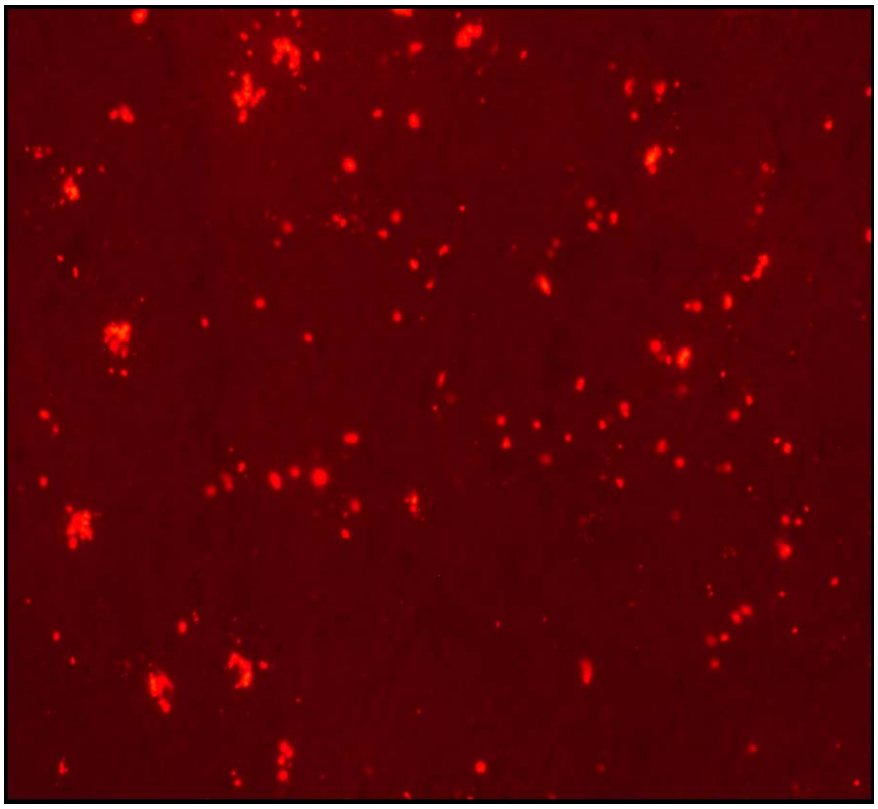

Figure 38 The above shows negative Alex Fluor/dsDNA probe fluorescence as visualised under 650 nm wavelength of light, on a spinal cord section taken from a female Biozzi (ABH) mouse that was showing peak EAE signs at relapse (x 40 objective).

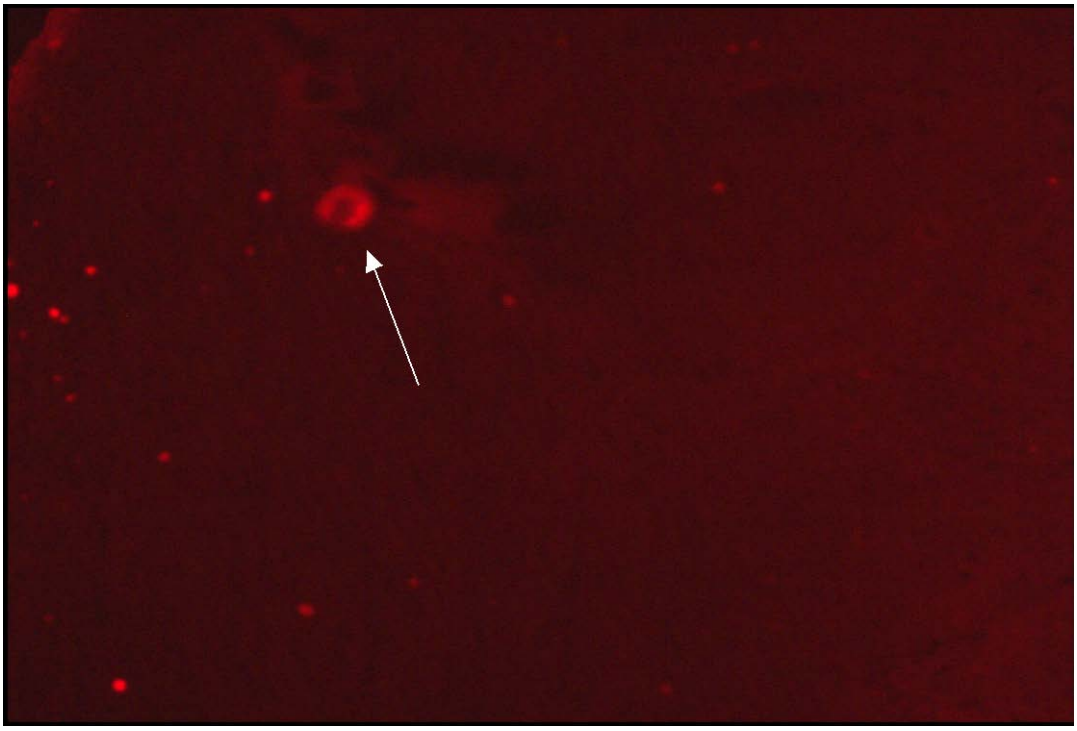

Figure 39 The above shows Alex Fluor/dsDNA probe fluorescence as visualised under 650 nm wavelength of light on a spinal cord section taken from a female Biozzi (ABH) mouse that was showing peak EAE signs at relapse (x 40 objective).

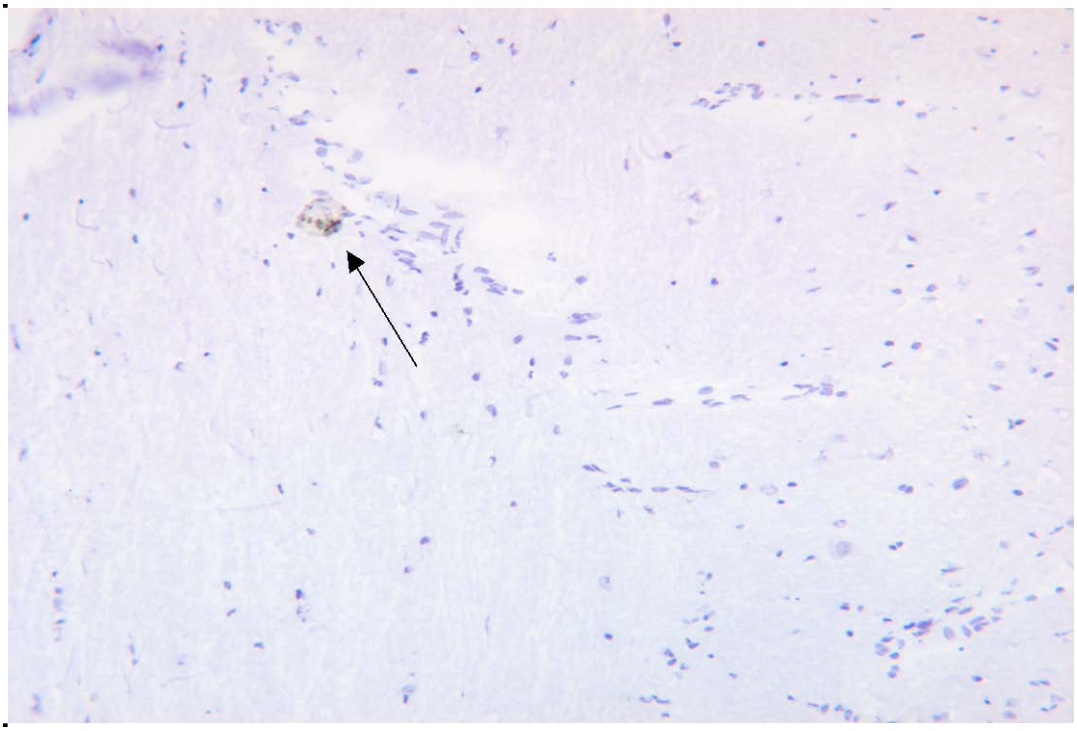

Figure 40 The above shows H & E staining on a serial section of a spinal cord as above, under (x 40 objective)

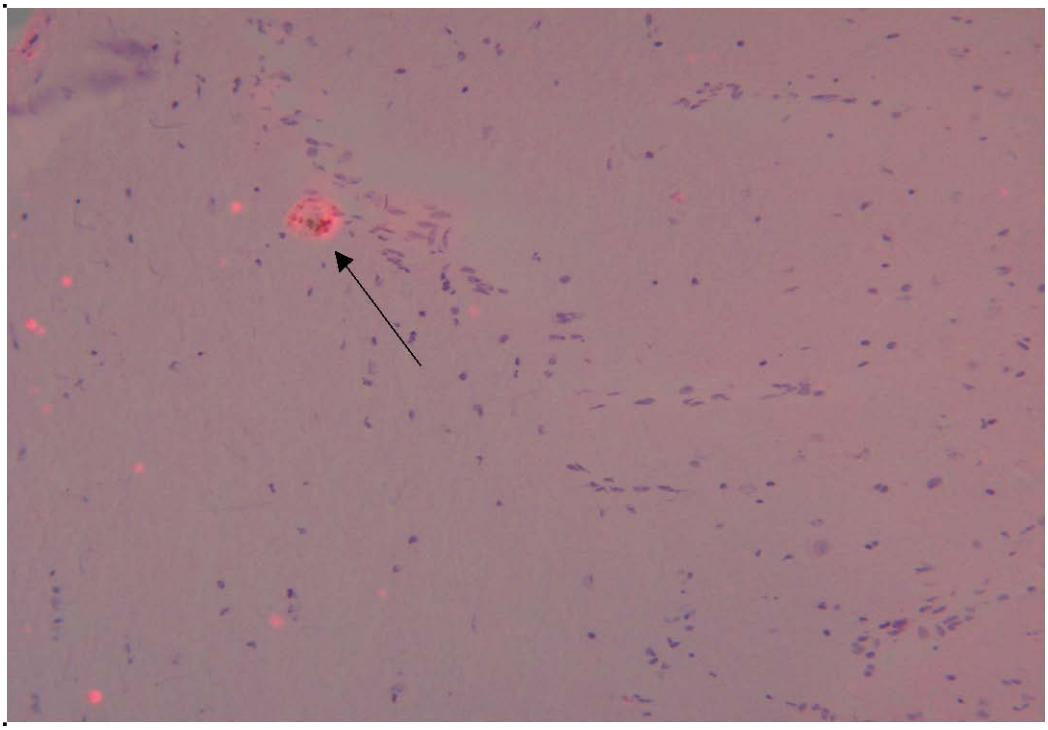

Figure 41 The above shows the composite of Alex Fluor/dsDNA probe and H & E on the same section of a spinal cord as shown in figure 40 (x 40 objective).

The fluorescent staining seen in figure 43 (indicated by the arrow) has that of a B cell appearance, however H & E and dual staining (figures 43 and 44) show the structure to be out of plane and have little cellular characteristics. The fluorescence seen was concluded to be a false positive.

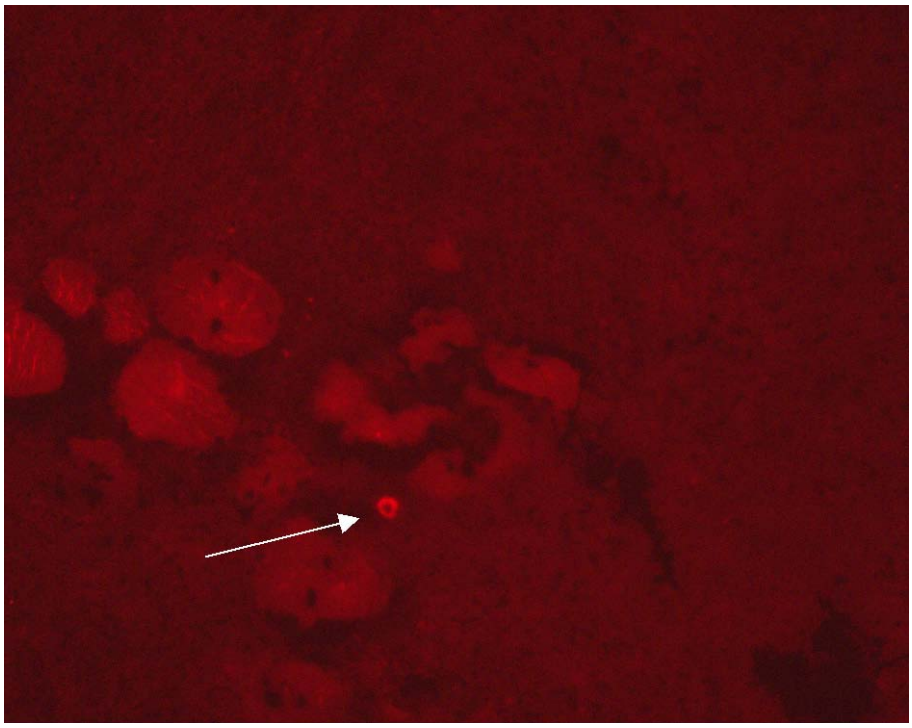

Figure 42 The above shows Alexa Fluor/dsDNA staining of a brain section taken from a female Biozzi (ABH) mouse that was showing peak EAE signs at relapse (x 40 objective).

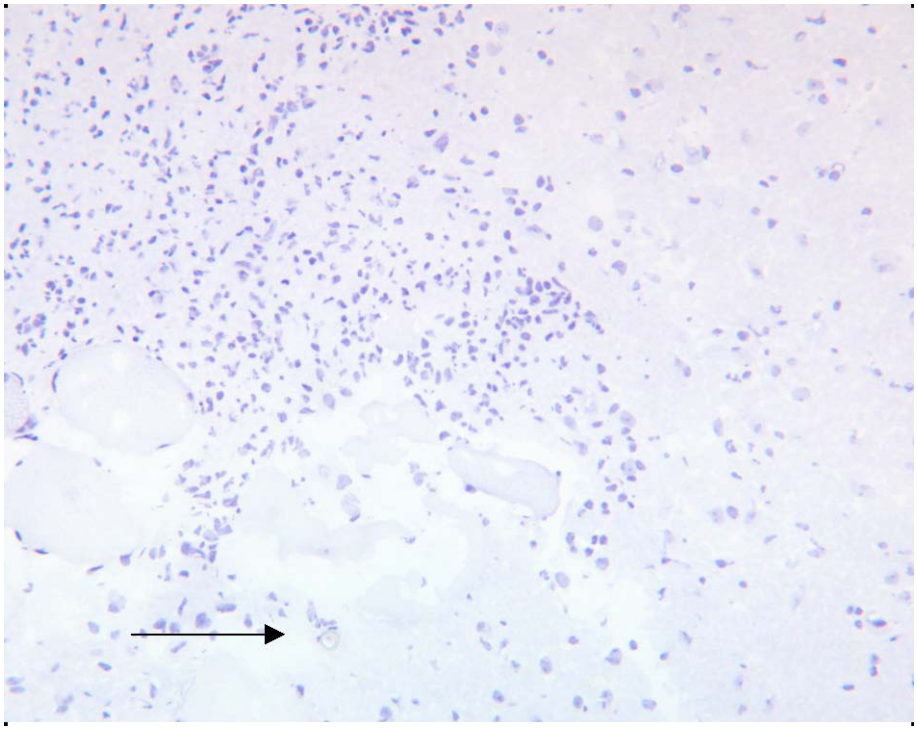

Figure 43 The above shows H & E staining of a serial section of brain as that shown in the figure above (x 40 objective).

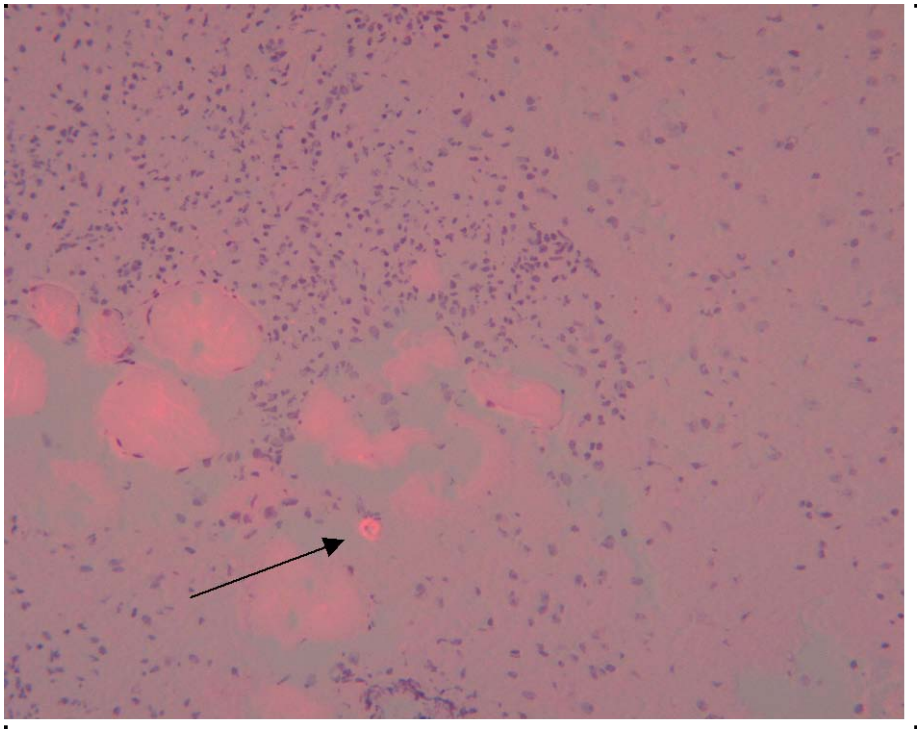

Figure 44 The above shows the composite of Alex Fluor/dsDNA probe and H & E on the same section of brain as shown in figure 43 (x 40 objective).

Figure 45 shows H & E staining of a section of spinal cord taken from a female Biozzi (ABH) mouse that was showing peak signs of EAE at relapse.

Figure 46 shows the same section of spinal cord visualised under red wavelength light following incubation with Alexa Fluor alone. The arrows indicate a number of discrete areas of slight fluorescence which from the pattern and size of the areas are comparable to nucleated cells, some of which appear to be forming sites of perivascular cuffing.

Figure 47 shows the same section of spinal cord as above, following staining with the Alexa Fluor/dsDNA probe. In this figure the areas of autofluorescence seen in figure 46 have disappeared. There are however three areas of fluorescence as indicated by arrows which do stand out from the section. These fluorescent areas due to their appearance size and location are thought unlikely to be true positives.

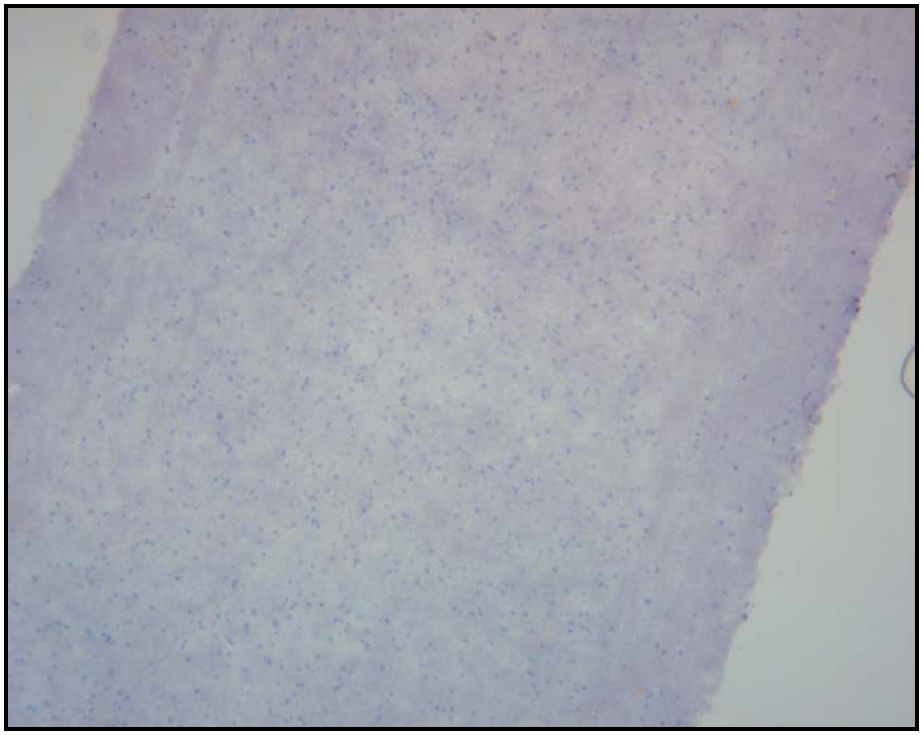

Figure 45 The above shows H & E staining of a spinal cord section taken from a female Biozzi (ABH) mouse, that was showing peak EAE signs at relapse (x 40 objective).

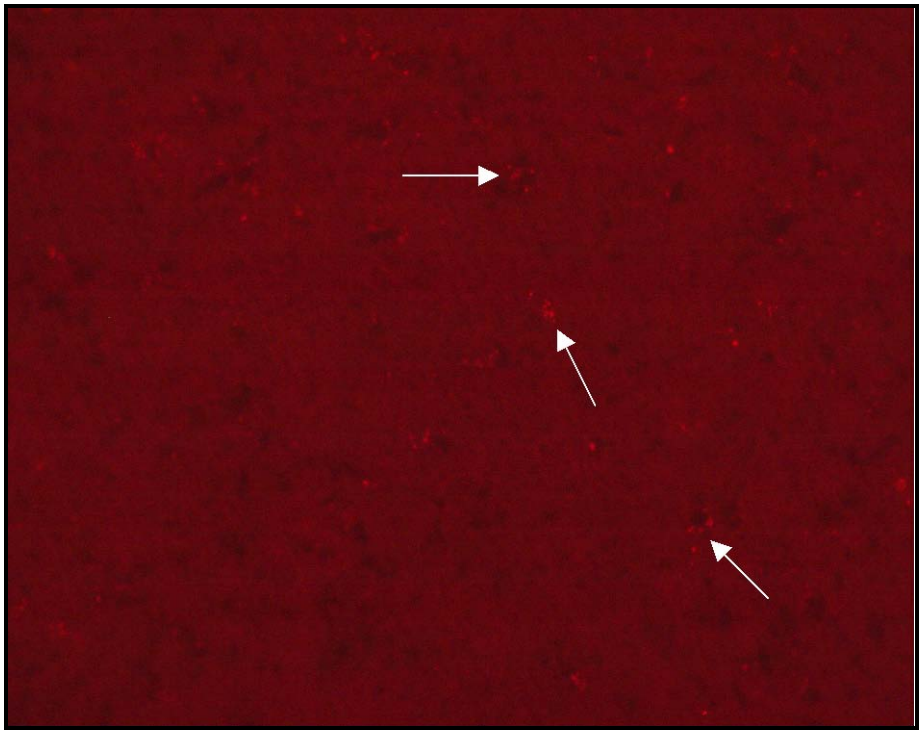

Figure 46 The above shows Alexa Fluor dye staining (in the absence of dsDNA) on the same section a spinal cord section (x 40 objective).

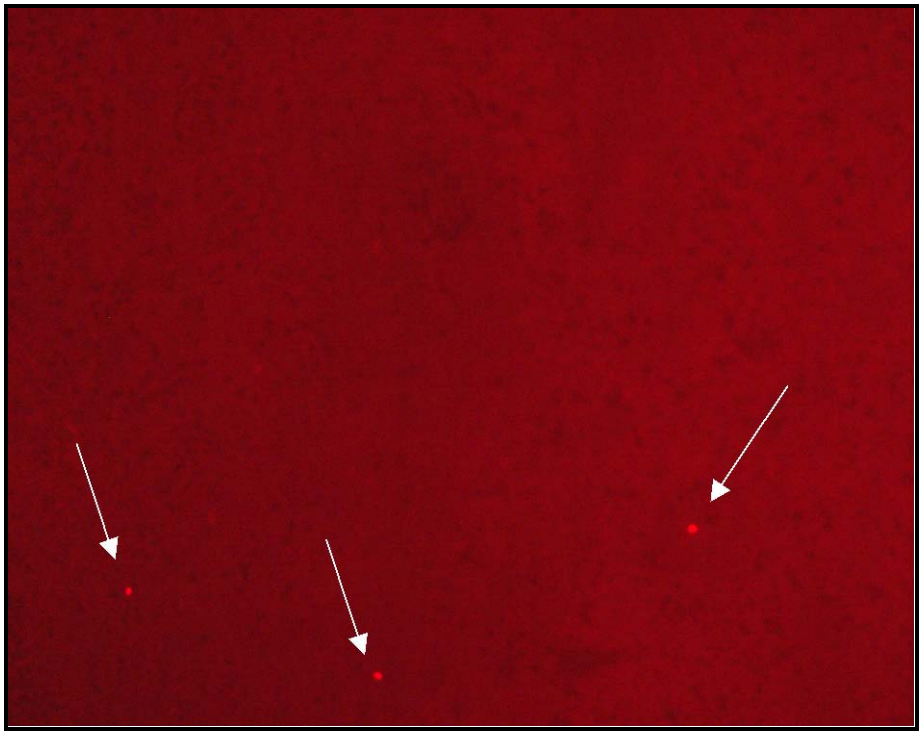

Figure 47 The above shows Alexa Fluor/dsDNA probe staining on the same section a spinal cord section as shown in figures 45 and 46 (x 40 objective).

3.6 Determination of anti-dsDNA antibodies in human MS tissue using Alexa Fluor/dsDNA probe

Due to this novel approach of detecting anti-dsDNA antibodies *in situ* it was extremely difficult to interpret whether the areas of fluorescence seen were false positives or true positives. There was no published reference information available to use for comparison purposes. In addition to this there was no indication from published literature to suggest that anti-dsDNA antibodies are found in animal models of EAE in the same way that they appear in MS, i.e. located around the edge of active lesions (plaques). A solution to this was to obtain human central nervous system tissue from a deceased MS patient *via* the MS Tissue Bank. A request was made for sections of brain and spinal cord tissue, which had been found to be positive for active lesions. The presence of active lesions being essential as this is where anti-dsDNA antibodies have been found to reside in MS.

Immunohistological staining of human spinal cord sections revealed no areas of positive staining for anti-dsDNA antibody. Brain sections however exhibited marked fluorescence, which was far more intense than that previously seen and when counter stained with H & E demonstrated good correlation with cellular bodies. Figures 48, 50 and 52, are not only good examples of the fluorescence seen, but also show good correction with a cell structure. It is possible that these areas of intense fluorescence are a result of autofluorescent pigment lipofuscin.

Figures 48 and 49 show the presence of anti-dsDNA antibodies (indicated by the arrows) on the outer edge of two separate areas. The larger of the two areas appears to be a blood vessel. The surrounding edge has a thick cellular wall, composed of endothelial cells. The smaller area has a less defined surrounding edge with fewer cells present, in addition there appears to be a small number of cells within the centre of the area. It is likely that this area is an active lesion.

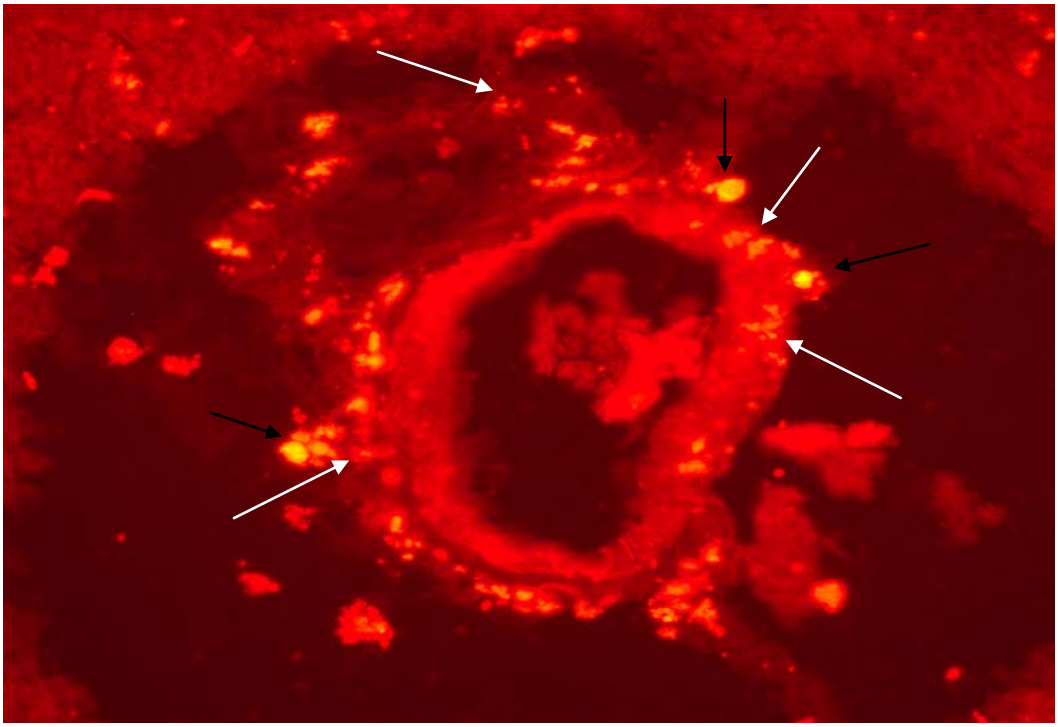

Figure 48 The above shows Alexa Fluor/dsDNA probe fluorescence, as visualised under 650 nm wavelength of light, on the brain tissue taken from a human MS patient (x 100 objective). The white arrows point to areas of positive staining, whereas the black arrows points to autofluorescence which is thought to result from lipofuscin.

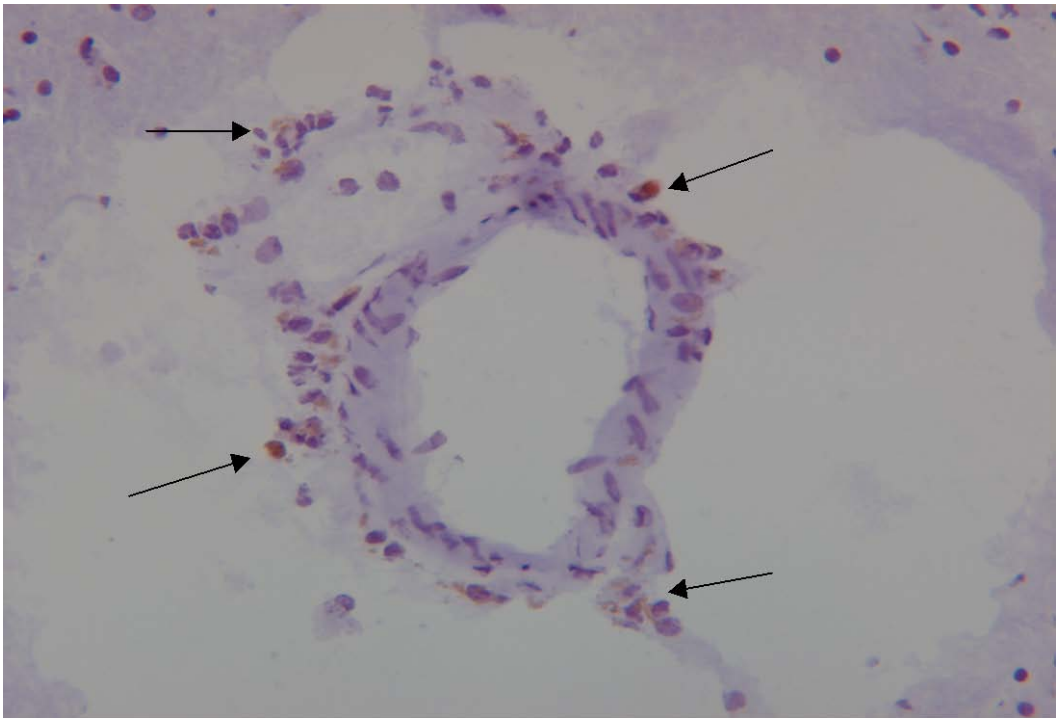

Figure 49 The above shows the composite of Alexa Fluor/dsDNA probe and H & E on the same section, same area of brain as above (x 100 objective). Arrows points to possible areas of lipofusion.

Figure 50 appears to show the location of fluorescence to be on the outer edge of an oval structure. H & E staining (figure 51) reveals the inner region of this structure to consist of what looks like to be a number of apoptic cells (indicated by the arrows). This finding suggests that the structure is an MS lesion, rather than a hole in the section. The absence of endothelial cells surrounding tis area rules out the possibility of this area being a blood vessel. The relatively large orange/yellow fluorescence is once again thought to be a result of lipofuscin.

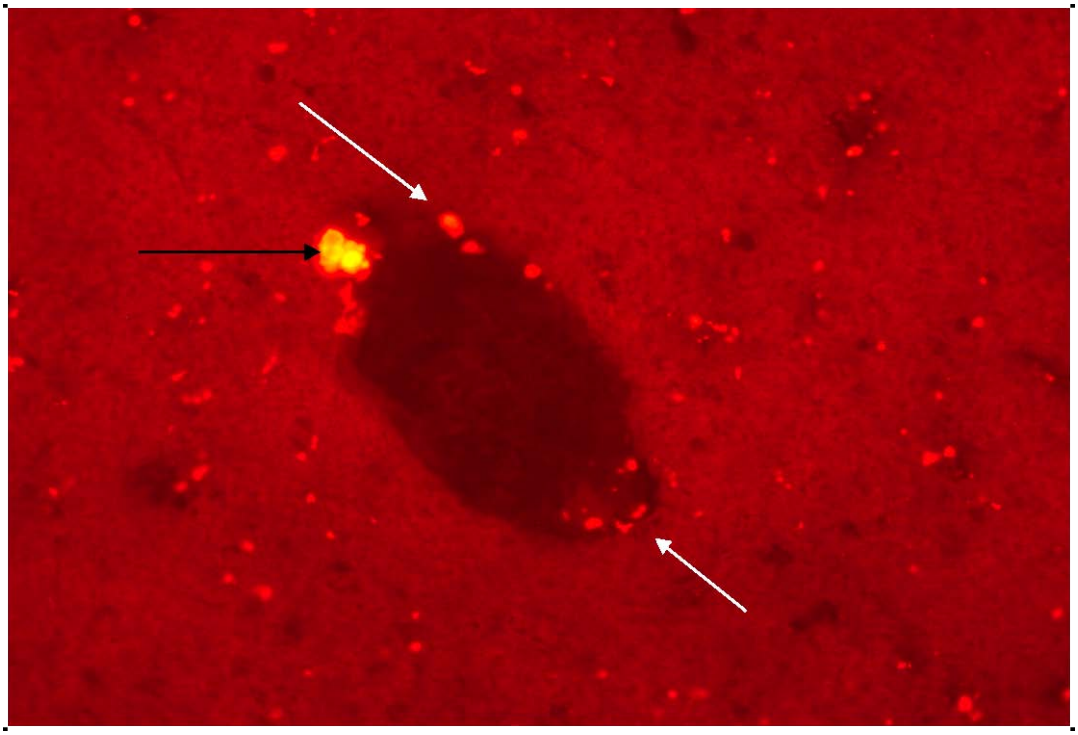

Figure 50 The above shows Alexa Fluor/dsDNA probe fluorescence, as visualised under 650 nm wavelength of light, on human brain tissue of a MS patient (x 100 objective). The white areas point to areas of positive staining, whereas the black arrow points to autofluorescence which is thought to result from lipofuscin

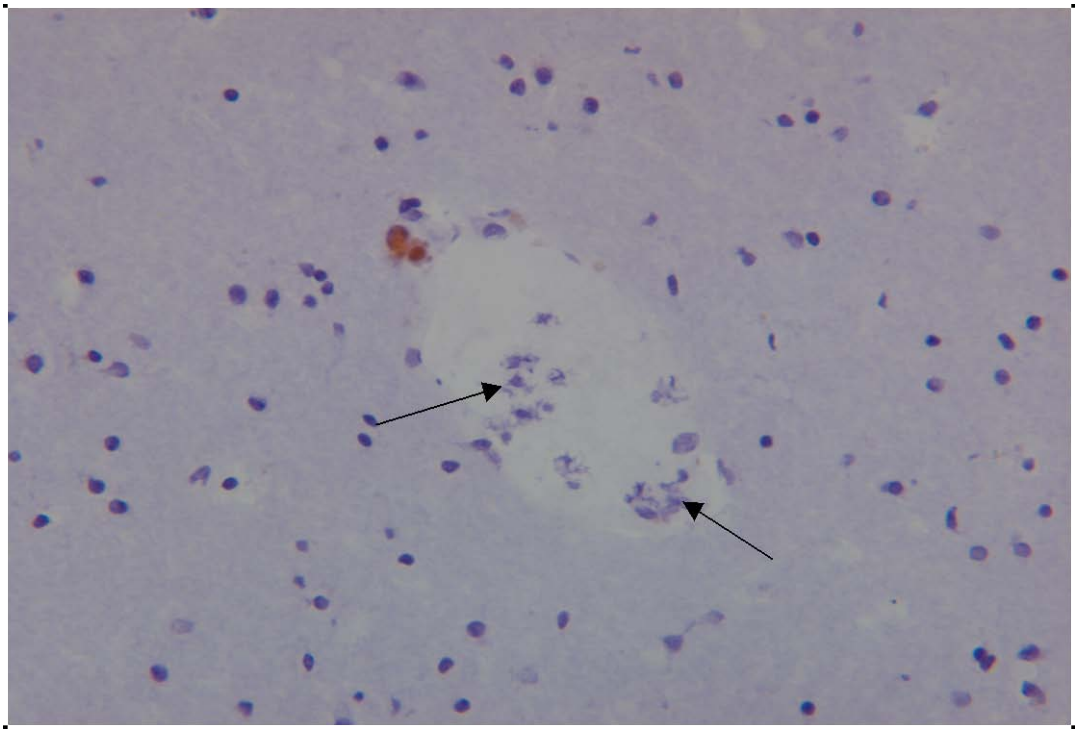

Figure 51 The above shows the composite of Alexa Fluor/dsDNA probe and H & E on the same section of human brain as above (x 100 objective). The arrows point to apoptotic cells.

Figures 52 and 53 show the presence of anti-dsDNA antibodies in what appears to be a collapsed blood vessel. Endothelial cells can be seen in figure 53 with positive staining for anti-dsDNA antibodies within this area.

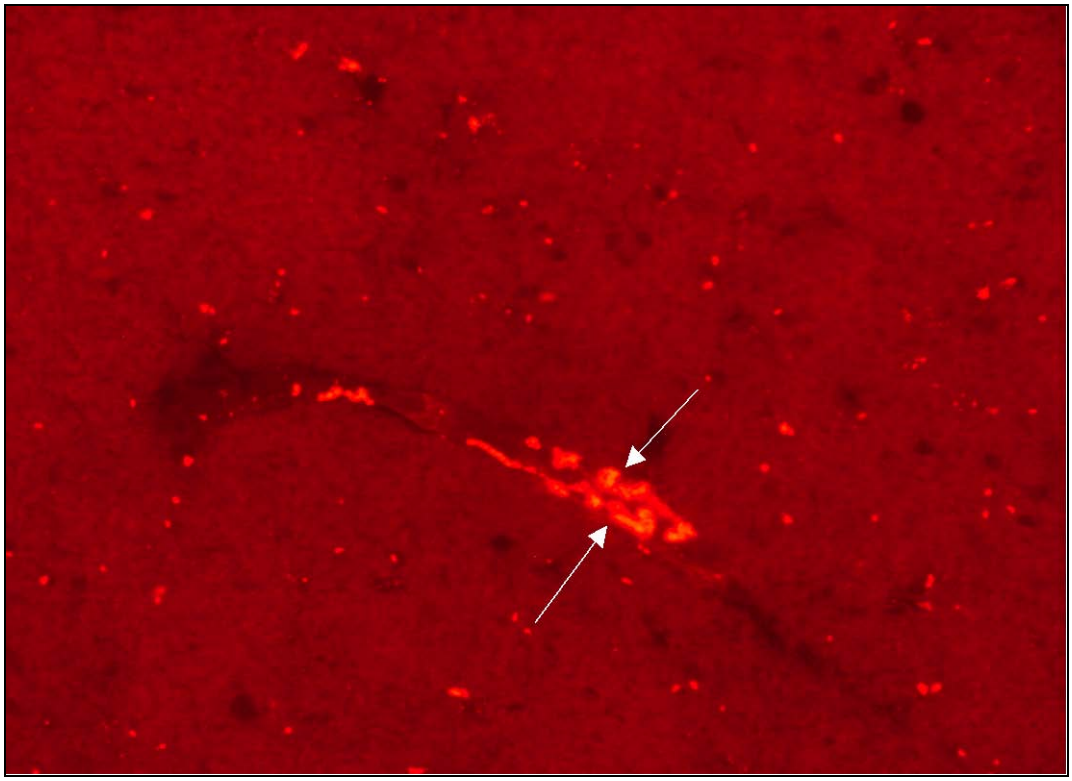

Figure 52 The above shows Alexa Fluor/dsDNA probe fluorescence, as visualised under 650 nm wavelength of light, on human brain tissue of a MS patient (x 100 objective). The white arrows point to areas of positive fluorescence.

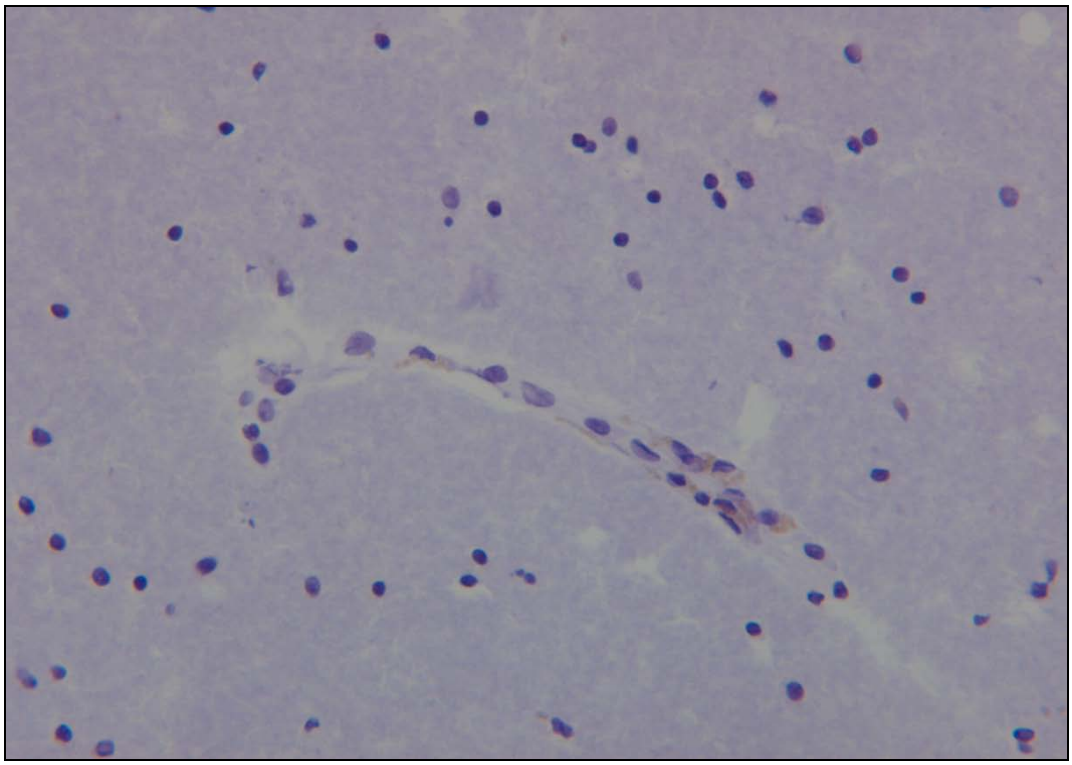

Figure 53 The above shows the composite of Alexa Fluor/dsDNA probe and H & E on the same section of human brain as above (x 100 objective).

4 CHAPTER 4: DISCUSSION

The aim of this project was to further understand the role of anti-dsDNA antibodies in multiple sclerosis and to determine if their presence is associated with pathogenicity.

Firstly and in order to do this, the validation of a number of methods for use in the detection of anti-DNA antibodies, IgG, IgM, C3 and B cells using the acute EAE model in the Lewis rat, relapsing/remitting EAE model in the Biozzi (ABH) mouse and in CNS tissue donated by an MS patient was performed.

The Lewis rat is an EAE susceptible strain of rat [Lassmann (1986)]. It is known to be a reproducible, reliable animal model of multiple sclerosis and a model that is particularly useful for examining the inflammatory phase of the disease [Lassmann (1986)]. This model was therefore initially selected to profile the disease process by evaluating the incidence of IgG, IgM, C3 and B cells in the parenchyma of the central nervous system and their relationship with clinical signs. Following inoculation with guinea pig whole spinal cord and the adjuvant carbonyl iron, the majority of animals displayed signs of EAE. Some animals however displayed signs to a greater severity than others. This led to the animals being categorised into two groups, successful and unsuccessful. Animals deemed to be successful displayed a marked EAE response, which occurred at the anticipated time following inoculation. Animals considered unsuccessful

displayed EAE signs to a lesser extent and in some cases such signs were absent. The first occurrence of signs following inoculation was also delayed in some cases when compared to the successful animals. The purpose of splitting the animals into these two groups was to see if the severity of clinical signs correlated with the incidence of specific histopathology parameters. For comparison, two further groups of animals were included in this study. The first of these groups was inoculated with adjuvant (carbonyl iron) alone, to rule out any potential effects caused by the adjuvant. The second of these groups consisted of animals that had not been treated with anything; these animals were included for reference purposes. In order to profile the progression of EAE, the animals were killed and sampled (blood, cerebrospinal fluid, brain, spinal cord) at five different stages during the disease process; onset, peak, plateau, remission and relapse (Day 34).

The use of carbonyl iron as an adjuvant compared to the typically used Freund's adjuvant, is a novel approach in EAE induction and was originally described by Levine and Sowinski (1980 &1989). It is an interesting adjuvant in that it has been found to induce signs of EAE when administered with an encephalitogenic agent in the Brown Norway rat, a strain of rat previously considered to be EAE resistant [Staykova *et al*. (2005)]. The Brown Norway rat has substantially higher nitric oxide levels upon immunisation with Freund's complete adjuvant compared to the Lewis rat. It is thought that administration of carbonyl iron may prevent the release of nitric oxide in Brown Norway, thus allowing for the induction of

EAE and if this is the case would suggest that high levels of NO have a protective effect against EAE [Staykova *et al*. (2005)].

In this study, although administration of carbonyl iron alone produced no clinical signs in rats, inflammatory infiltrates were present throughout the spinal cord. The greatest difference in the incidence (frequency of areas of positive staining) of IgG, IgM, C3 and B cells between the carbonyl iron control and CNS-treated animals occurred at the sites of perivascular cuffing. At these sites IgG, IgM, B cells and C3 were negligible in the carbonyl group compared to the incidence seen in the spinal cords of animals successfully developing EAE. This finding alone suggests that the area of interest with regards to EAE pathology in the Lewis rat is at the perivascular cuffing sites. To further support this theory, the results indicate that it is at such sites that a distinct correlation between the degree of inflammatory infiltrates and the severity of clinical signs can be seen in animals inoculated with CNS material. Animals successful in EAE development had a higher incidence of inflammatory infiltrates compared to those animals displaying EAE signs to a lesser extent (unsuccessful) at these sites.

Results show that perivascular cuffing in the carbonyl control animals remained at a low level throughout the study. The incidence of perivascular cuffing was found to be greatest in successful EAE animals and slightly less in the unsuccessful EAE animals. Incidence for both groups peaked at the time that EAE signs had peaked (unsuccessful

animals) or reached a plateau (successful animals), thus demonstrating a close correlation between PVC and EAE signs. The incidence of PVC was seen to decline as EAE signs diminish.

The correlation between inflammatory infiltrates and clinical signs is also true in multiple sclerosis, where in the majority of people active MS is associated with inflammatory infiltrates in the parenchyma of the central nervous system, whereas people in the inactive stage of the disease exhibit very little in the way of such a response [Khan *et al*. (2008]. In addition, active MS lesions have a plasma cell presence, which produce mainly IgG antibodies and to a much lesser extent IgM; PVC is also a well-recognised feature of MS. These characteristics are also the case in the Lewis rat EAE model as demonstrated in this project. The similarities between MS and the acute EAE model in the Lewis rat supports the use of this animal model in MS research, in particular as a model for the discovery of novel drugs, which are intended to suppress or modify the inflammatory response. This model however is not appropriate as a research tool if the area of interest is demyelination.

Activation of complement in MS is thought to be a primary cause of demyelination, possibly as a consequence of prolonged damage indirectly caused by C3 activation resulting in a release of self-antigens e.g. DNA histones or MBP. However, in the Lewis rat despite the presence of significant C3 activation, demyelination is not seen in this model [Antel *et al*. (2005)]. This would suggest that there are other mechanisms involved

in the demyelination process in addition to, or in the absence of complement.

Activation of C3 results in the cleavage of the molecule to form C3b and C3a. It is C3a that is responsible for causing vascular permeability, which in MS and EAE is key to allowing inflammatory infiltrates to pass through the blood brain barrier and thus potentially causing destruction within the CNS. The release of C3a also triggers phagocyte and macrophage recruitment. C3b tags the 'foreign' body so to increase the selectivity and efficiency of the inflammatory response. Some studies have demonstrated that complement activation is necessary for the development of EAE and this has been supported by studies using C3aR-deficient mice (mice deficient in the anaphylatoid receptor C3a). These studies have found that by preventing C3, EAE symptoms can be alleviated or prevented [Boos *et al*. (2005)]. Other studies however have found that that C3 (and C4) are not involved in EAE for which the encephalitogenic agent is the immunodominant epitope of MOG, peptide 35-55 [Calida *et al*. (2001)].

The results obtained from the Lewis rat EAE model in this study suggest that overall C3 incidence has little or no role in the development of EAE clinical signs. The overall incidence of C3 was comparable between animals successful in developing EAE and for those animals inoculated with carbonyl iron alone, which did not exhibit any EAE clinical signs. The presence of C3 at sites of PVC however did show a close correlation with

clinical signs, with there being negligible detection at PVC sites in the carbonyl iron control animals. In addition, it appears as if the location of C3 is of greater importance than the overall magnitude of this molecule. C3 incidence at peak EAE was approximately 4-fold less at PVC sites than at non-PVC sites.

In the EAE model in the Lewis rat the pathological role of C3 is likely to relate to its ability to increase vascular permeability, causing areas of localised accumulation of inflammatory infiltrates around blood vessels, that is, perivascular cuffing.

The question is, what causes the increase in C3, which is seen within the parenchyma of the central nervous system. Is it in response to autoantibodies or inflammation in response to the administration of carbonyl iron?

In this study the peak incidence of IgG antibody, C3 and B cells at perivascular cuffing sites occurred one-step behind the first occurrence of marked EAE signs (peak). That is, maximal infiltration was seen during the effector stage of the disease, at the time when the first marked signs of EAE (substantially limp tail) were seen. This could be considered to be the time when the lesions were active, i.e. causing damage to the CNS. Following ‘peak’ EAE the incidence of these parameters decreased sharply. This supports the hypothesis that the presence of at least one of the above parameters (IgG, C3, B cells) has a potential pathogenic role in

EAE. The only parameter not following this trend was IgM, for which the incidence was relatively low at PVC sites at all time-points with the exception of the plateau time-point where levels sharply increased. There are two possible reasons for this, the first being that following the active phase of the EAE episode IgG antibodies have disappeared and are replaced by non-pathogenic IgM antibodies 'clearing-up' following the peak inflammatory phase of EAE by opsonising apoptic cells and therefore reducing the risk of further tissue damage. The second explanation is that their presence may be in response to an antigen which is different to the one initially driving the response. It is possible that breakdown products released as a result of damage caused by the initial inflammatory attack have caused a secondary response. Such breakdown products could include DNA and histones, which could form autoantigens leading to further destruction of the CNS. If this is the case then subsequent attacks would result in increasing levels of these antibodies. Studies have found that some IgM antibodies that are specific for CNS antigens potentiate remyelination [Warrington and Rodriguez (2007)]. It is also worth commenting on the peak incidence of IgM throughout the spinal cord parenchyma (i.e. not localised to PVC sites) which occurred at the time of EAE onset. It is possible that this peak was in response to the encepahlitogenic agent/adjuvant being treated as an invading microorganism. This may explain this early, un-localised effect.

The incidence of B cells in sections of spinal cords obtained from animals successful in EAE development sampled at peak, was found to be notably

higher than the incidence of C3 and IgG antibodies at this time-point. This increased presence may suggest a pathogenic role for B cells in this model. Studies have suggested that B cell pathogenesis is through demyelination rather that its effects on inflammation [Svensson *et al.* (2002)], however, in the EAE model in the Lewis rat this is unlikely to be the case, as demyelination is not seen. Alternatively, it may be that B cells have a role in limiting EAE duration. Studies using B cell deficient mice have found that EAE in these animals is a long-term chronic disease compared to wild-type mice that, in a short period of time recover completely [Wolf *et al*. (1996)].

This study has shown there to be a correlation between leukocyte infiltration, perivascular cuffing and EAE severity. These findings may be of some significance with regards to the following alternative hypothesis of MS pathogenesis [Reik (1980)].

It has been found that vascular changes following various infections and some vaccinations are often associated with demyelination [Poser (1978)]; this is known as disseminated vasculomyelinopathy. It has been proposed that in diseases of sudden onset, marked severity and short duration [Field (1975)] as in the EAE model in the Lewis rat, vascular changes may be more important than demyelination in the disease process.

The hypothesis for disseminated vasculomyelinopathy assumes that following infection antibodies form and bind with the antigen, forming

circulating immune complexes. If these complexes are of an appropriate size and contain the correct proportion of antigen to antibody they can cause the systemic liberation of vasoactive substances. This results in an increase in vascular permeability with complexes becoming focally trapped in vessel walls, complement is activated and inflammatory cells accumulate releasing proteolytic enzymes thus causing tissue injury [Cochrane and Dixon (1978)]. If this is the case then demyelination may occur as a by-product of this event.

This theory supports the possibility that demyelinating antibodies are not the key pathogenic agents in MS and that they are potentially a by-product. To support this further, it should not be overlooked that despite MS pathology focusing on perivenous lymphocytic infiltration and demyelination within the CNS white matter [Carpenter and Lampert (1972); Oppenheimer (1976)], there are many other pathological reactions occurring at all levels within the nervous system. These range from perivascular cuffing, lymphocytic infiltrates and endothelial proliferation to vessel wall necrosis, fibrinoid degeneration, infiltration of neutrophils and eosinophils and perivascular haemorrhage [Carpenter and Lampert (1972); Oppenheimer (1976); Hart and Earle (1975); Miller *et al*. (1956); Reik (1980)] and occur in arterioles and capillaries as well as venules [Carpenter and Lampert (1972); Reik (1980)]. These occurrences are frequently more common in the cerebral cortex, basal ganglia and grey matter of the brain stem and spinal cord (in the centre) than in white matter [Carpenter and Lampert (1972); Hart and Earle (1975)]. It must

also be remembered that in addition to demyelination, axons are often substantially affected in MS and a clear differentiation between perivascular demyelination and necrosis, which can cause the same clinical symptoms, has not always been made [Hart and Earle (1975); Reik (1980)].

It is possible that changes in vascular permeability and perivascular inflammation may alter the antigenicity of myelin or release antigens previously sequestered by the blood brain barrier. The cell-mediated immune response could then maintain the damage initiated by the original vascular injury and this would explain the presence of antibodies to myelin. In MS, the nervous system appears to be preferentially targeted by immune complex mediated vascular injury. This may in part be explained by the fact that any resultant permeability is going to be more serious in the nervous system which is protected by the blood brain barrier, than in other organs where the vascular bed is already permeable [Reik (1980)].

If the above hypothesis proves to be correct, then this would go some way in explaining the increased incidence of IgG, B cells and C3 in regions of perivascular cuffing and the appearance of EAE signs in the absence of demyelination in the Lewis rat. It would also provide a parallel with SLE where immuno-complexes are recognised as having a pathogenic role with anti-dsDNA antibodies being of major importance.

In addition to histopathology, serum and CSF samples collected from Lewis rats at each of the sample times (onset, peak, plateau, remission and relapse / Day 34) were analysed for the presence of anti-dsDNA and anti-ssDNA antibodies using an ELISA. Limited CSF volume (approx. 0.1 mL) was the main reason contributing to the incomplete data set in this study.

Although the ELISA was the assay of choice for this study and one proven to be of sufficient sensitivity [Alnaqdy *et al*. (2007)], there are other assays available for anti-DNA antibody detection in CSF and sera. Frequently used methods are telomeric DNA sequence recognition and binding [Salonen. (2002)], isoelectric focusing (IEF) and immunoblotting [Fortini *et al*. (2003)]. In the clinical setting, sufficiently large volumes of CSF can be obtained and as such volume is rarely a limiting factor. Typical volumes required for analysis are 0.5-2.5 mL of CSF and approximately 1 mL of sera, depending on the tests to be performed. [Laboratory Corporation of America]. In the clinic these volumes are not only possible, but can also be obtained repeatedly if necessary. In this study, 0.1 mL was the average volume of CSF collected and this was a terminal sample which meant that an insufficient or contaminated sample could not be replaced.

Analysis of the data obtained in this study showed there to be a statistically significant increase in anti-dsDNA antibody in the sera from CNS immunised animals sampled at the time of disease onset when

compared to the associated carbonyl control group. No other significances were observed.

The ELISA data for sera samples showed that anti-dsDNA and anti-ssDNA antibodies remained relatively constant at all sample time-points and this was true for both the carbonyl iron control animals and the EAE animals. It is not surprising to find low levels of circulating anti-DNA antibodies in the sera and this is found to be the case in people.

In contrast, the ELISA data for CSF samples showed a distinct trend, with a peak in anti-dsDNA antibodies at the plateau sample time-point and a trough at the same time-point for anti-ssDNA antibodies. Although samples from both the controls and EAE animals follow the same trend for both antibodies, the greatest effect can be seen for the samples taken from the EAE animals. The interesting point here is that at the time when EAE signs have reached a maximum, anti-ssDNA antibody levels are at their lowest level, whereas anti-dsDNA antibodies are at their highest level. The decrease in anti-ssDNA antibodies at this time-point could be due to them binding to DNA from damaged tissue during the peak phase of the disease. This is to be expected and is part of the clearing-up process whereby anti-ssDNA antibodies are forming immunocomplexes for disposal. This drop in antibody level has also been reported by Stetler *et al.* (1985) during their research into the correlation of antibody production with delayed onset of Lupus-like disease in MRL +/+ mice.

As these results are reflective of a single acute EAE episode, a greater antibody response leading to an uncontrolled immune response was to be expected in a relapsing/remitting model of EAE. In a relapsing/remitting EAE model it is thought that the controlled immune response as seen in cases of bacterial infection is compromised. Under normal conditions IgM anti-ssDNA antibodies are produced and the switching to high affinity pathological anti-dsDNA antibodies is prevented. However, if this block for some reason is removed, then these antibodies are produced, leading to their predominance in the CSF and contributing to an uncontrolled autoimmune state.

Regardless of the results obtained, the differences in antibody levels were slight and the data too limited to reach any meaningful conclusions. In addition, no CSF data was available for animals sampled at peak EAE. This was due to insufficient sample volumes obtained or the CSF becoming contaminated with blood during the collection procedure.

It is possible that the low level of anti-dsDNA antibodies detected may be due to these antibodies being compartmentalised within the parenchyma and as such not free in CSF. Alternatively it is likely that a single episode of EAE is not sufficient to produce a marked antibody response. This response would be much increased with each subsequent episode or in a model of chronic progressive EAE. This has been found to be the case in a Russian study [Ershova *et al*. (2003)] of MS patients where an

association between increased anti-dsDNA antibody titre and the level of disability was observed.

In this project anti-DNA antibody levels in cerebrospinal fluid from the Lewis rat were found to peak with the plateau phase of the disease and decline in remission. As successive phases of relapse and remission result in successive rounds of antibody selection this finding suggested that in order to investigate these antibodies a relapsing model of EAE was required. The evidence for the importance of a relapsing/remitting model comes from the experience in SLE where the relapsing/remitting phase of the disease leads to multiple rounds of antigen selection [Ehrenstein *et al*. (1993)]. Given that MS is a relapsing and remitting disease the same may occur in MS.

In addition to finding a relapsing/remitting EAE model, the issues relating to insufficient volumes of CSF placed a greater emphasis on developing a technique which would enable anti-dsDNA antibodies not only to be detected, but also their position within the central nervous system to be visualised. In the early stages of disease, the generation of autoantibodies that are the result of multiple rounds of antigen stimulation may be difficult to detect, as such detection of the presence of anti-dsDNA antibody secreting cells was considered to be an alternative approach [Borg *et al*. (1990)].

Methods often used for the detection of anti-DNA antibodies are the microtitre plate assay [Mouratou *et al*. (2002)], enzyme-linked immunosorbent assay (distinguishing IgG and IgM anti-ssDNA and anti-dsDNA), *Crithidia luciliae*, a nitrocellulose filter assay, the Amersham kit, and the radioimmunoassay [Isenberg *et al*. (1987)]. Literature searches revealed no documented method for the detection of anti-DNA antibodies *in situ,* as such the probes developed for use in this project are considered to be a novel approach for visualising anti-dsDNA antibodies.

It was hypothesised that if DAPI was allowed to bind to dsDNA the resulting mixture could be used as an anti-dsDNA antibody probe. The DAPI/dsDNA probe was incubated with sections of rat spinal cord, with a view that if anti-dsDNA antibodies were present then binding would occur resulting in positive fluorescence.

DAPI has a peak excitation wavelength of 340 nm and an emission maximum at a wavelength of 488 nm. However, when bound to dsDNA the excitation peak occurs at a longer wavelength of approximately 360 nm. In this project DAPI was initially bound to dsDNA and once incubated with tissue sections was potentially further bound to anti-dsDNA antibodies. Although not documented, the possibility of this effect further increasing the excitation wavelength was considered. When viewed under the recommended ultraviolet light, marked fluorescence was observed (figure 36). The intensity of this effect, which was most likely the result of free DAPI binding to nucleic acids was so great that the detection of anti-

dsDNA antibodies was thought to be unlikely. A decision was taken to view the tissues sections, once incubated with the DAPI/dsDNA probe under a much longer wavelength of light in order to visualise a more subtle effect. Visualisation subsequently took place under green light (wavelength of approximately 510 nm). Patterns of fluorescence were seen under this light which where thought to relate to positive staining (figures 28, 33 and 35). Based on these results the DAPI/dsDNA probe sections were viewed under green light. For interests sake and based on no particular scientific rationale sections were also viewed under red light. Aschar-Sobbi *et al*. (2008) developed a protocol for the detection of the signalling molecule polyphosphate (poly-P) whereby the optimal excitation wavelength for the DAPI-ploy-P complexes was found to be 550 nm (within the green light range). By using this longer wavelength fluorescence from free DAPI and DAPI/DNA was minimal, thus allowing for the visualisation of poly-P.

Although the ELISA for the detection of anti-dsDNA and anti-ssDNA antibodies was found to be inconclusive, initial investigations using the DAPI/dsDNA probe suggested the presence of anti-dsDNA antibodies in the spinal cord of rats in this model.

The DAPI/dsDNA probe produced a number of potentially positive results when incubated with Lewis rat spinal cord sections. Based on the disease profiling results, spinal cords taken from successful animals at peak EAE were used, this being the time when IgG antibodies were at their greatest

incidence. Potentially positive staining was found in a number of sections. Some of these were concluded to be artefacts, either because the area of positive staining was too large to be any cell type or because they were out of the plane of the tissue section. The most encouraging positive staining had the appearance of a number of relatively large cells clustered together with positive DAPI staining surrounding each cell (figure 28). Following a literature search a similar picture (figure29) was found that showed plasma cells containing Russell bodies [reference not available]. Although scales are not available for either figure, the figures are very similar in appearance and the association of Russell bodies with chronic inflammation would not put them out of place in this model [Kopito & Sitia (2000); Pelc *et al*. (1981); Tsunoda *et al*. (2005)].

Russell bodies are thought to be an accumulation of condensed immunoglobulins in the endoplasmic reticulum cisternae probably resulting from a disturbed secretion [Pahl & Baeuerle (1997) and Kopito & Sitia (2000)]. Although they can be found in many cell types, they frequently occur in plasma cells and may develop as a response of the cell trying unsuccessfully to get rid of somatically mutated stable unsecretory molecules [Pahl & Baeuerle (1997) and Kopito & Sitia (2000)]. The retention of immunoglobulins in Russell bodies suggests that the mechanism of protein transport in those cells affected is incompetent resulting in the accumulation in dilated endoplasmic reticulum cisternae of proteins that are neither degraded nor secreted [Pahl & Baeuerle (1997) and Kopito & Sitia (2000)]. The accumulation of immunoglobulins may be

so great that it results in the endoplasmic reticulum overload response which results in the induction of cytokines followed by inflammation and apoptosis [Pahl & Baeuerle (1997) and Kopito & Sitia (2000)]. Russell bodies may be pathogenic with regards to inflammation and apoptosis or protective by maintaining the secretory pathways [Valetti *et al.* (1991)]. If indeed the structures seen in figure 28 are plasma cells containing Russell bodies their presence with regard to pathogenicity is unknown, other than inflammation being a primary characteristic of EAE and Russell bodies being associated with such events.

The results from the Lewis rat and DAPI/dsDNA probe experiments were encouraging, but improvements to the methods used were considered to be necessary if conclusive results were to be obtained. Two approaches were taken. The first was to refine the probe used for the detection of anti-dsDNA antibodies and the second was to use an EAE animal model for which the disease process was closer to that seen in people; this being a relapsing/remitting form of EAE for which demyelination is a characteristic. The DAPI/dsDNA probe was replaced by an Alexa Fluor/dsDNA probe. The principal was the same, just the fluorescence agent differing and the light under which the tissue sections were examined (red light). The Alexa Fluor/dsDNA probe was initially tested in Lewis rat spinal cord sections with little success and testing was stopped until spinal cord from a relapsing/remitting model of EAE was available.

The Biozzi (ABH) mouse was selected as the species of choice for the relapsing/remitting EAE model. This mouse is a frequently used strain for this type of testing and is well documented in published literature. Many immunological animal models can be difficult to establish to such an extent that reproducible, reliable results are obtained. This was certainly found to be the case in this project. Eight consecutive attempts over a period of 21 months were made to validate a reliable and reproducible EAE model in the Biozzi (ABH) mouse. The differences in the protocols used revolved around the route of administration of the encephalitogenic agent (intradermal injection into the base of the tail, intraperitoneal administration and subcutaneously into the flank), sex of the animals (males or females), the encephalitogenic agent (mouse spinal cord, guinea-pig spinal cord, bovine MBP or murine MBP) and the adjuvant used (FCA, FCA plus *Mycobaterium butyricum* or carbonyl iron).

None of the protocols used proved to be successful, despite being used as standard models for EAE [Liblau *et al*. (1997), Heremans *et al.* (1999) and Asensio *et al*. (1999)]. EAE signs were only seen in 3/18 female mice following inoculation by the intradermal route into the base of the tail with homogenised Biozzi (ABH) mouse spinal cord, 2/6 female mice inoculated intradermally into the base of the tail with murine MBP and 1/5 female mice inoculated intradermally into the base of the tail with homogenised CD-1 mouse spinal cord. None of the animals that initially showed EAE signs relapsed following remission.

The results suggest that females mice are more prone to EAE and this is supported by Baker *et al.* who found that EAE susceptibility in Biozzi (ABH) mice was increased by a major locus in chromosome 7 [Baker *et al*. (1995); Sinha *et al*. (2008)] and also that females tend to have a stronger Th-1 mediated immune response [Sinha *et al*. (2008)]. With some refinement, intradermal administration into the base of the tail of spinal cord homogenate using FCA/FIA has the potential to induce EAE in the Biozzi (ABH) mouse.

The reasons as to why so few animals responded and the complete absence of relapse may be due to a number of factors. Group housing of the animals may increase stress levels. This is likely to have affected males more than females due to an increase in bullying of cage mates seen in male mice as they get older. Stress causes the release of corticosteroids [Joéls & van Riel (2004)], which has immunosuppressant properties and potentially the ability to prevent EAE. However, individually housing animals in this project did not cause any of the animals to develop EAE. Another possibility is interference from hormones in female animals. Studies [Kim *et al*. (1999)] have shown oestrogen to have a protected role against EAE development. Teuscher *et al.* (2004) investigated the effect of gender, age and season on EAE susceptibility in mice. They found that clinically, the chance of developing EAE signs increased by 4% for each increasing week of age and that there was a 90% increase in the chance of developing EAE when immunised in the summer time compared to the spring and winter months (February – May). In this study the age of the

animals ranged between 5 – 12 weeks of age at the time of immunisation, with there being no effect on EAE susceptibility. Immunisation took place of a period of 21 months at various times in the year and did not appear to increase/decrease EAE susceptibility. Finally, it is possible that the Biozzi (ABH) mice were not strictly antibody high. Antibody high (ABH) Biozzi mice elicit high antibody titre response following immunisation with an encephalitogenic agent and have a high susceptibility to EAE compared to antibody low mice for which EAE susceptibility is much reduced [Baker *et al.* (1990)]. The difference in antigenic response is thought in part to be attributed to genetic difference in macrophage activity. Interestingly, antibody high and low Biozzi mice respond differently to parasitic infection but this is not related to the degree of antibody titre elicited [Male *et al*. (2006)].

In the interests of moving this project forward and to limit the number of animals being used, spinal cord and brains were sourced from a lab which has a proven record of the EAE relapsing/remitting Biozzi (ABH) mouse model. Alexa Fluor/dsDNA staining was performed on the spinal cord and brains of mice which had been killed and sampled during a second episode of EAE. Although slight positive staining did occur on a section of spinal cord, dual staining techniques using Alexa fluor/dsDNA and H & E indicated that the staining observed was an artefact of the slide preparation procedure. A limitation of being the recipient of donated mouse tissue was the lack of control with regards to the time at which the animals were sampled. Instructions were given to sample the animals at

the time of peak EAE, as soon as the animals exhibited a completely limp tail during the second EAE episode. However, as histological signs return to normal soon after peak, any delay in sampling, even by a day would make detecting lesions more unlikely.

The lack of a positive control was a problem throughout this project and in its absence lengthy investigations were performed on areas of positive staining in order to determine if they were true positives. In addition to this there was no literature available that suggested that anti-dsDNA antibodies are present in the animal models used. The next step was to obtain human tissue from a human who had suffered from MS. Such tissue was obtained from the MS Tissue Bank. A request was made for sections of spinal cord and brain which had active lesions. Active lesions were important as anti-dsDNA antibodies are known to be present on the outer edge of such lesions [Williamson *et al*. (2001)]. Such sections were thought to be the best chance of seeing positive staining which would prove that the probes work *in situ*.

Brain and spinal cord sections were examined for the presence of active lesions by the MS Tissue Bank prior to dispatch, using Luxol fast blue/MHC class II. The presence of a large lesion with immune activity on the lesion edge was found in the sections of brain tissue, but less clear staining was found for the sections of spinal cord. The human tissue samples were donated by a patient suffering from chronic MS. The patient was suffering mild symptoms approximately 10 years prior to his death

from the disease. The disease took on a chronic progressive form which would have led to raised antibody titres, which probably played a significant pathological role during the late stages of the disease.

Alexa Fluor/dsDNA probes incubated with spinal cord tissue from the MS patient failed to produce positive staining. This was not unexpected, as information provided by the Tissue Bank indicated that staining of the spinal cord block had been found to be less clear compared to that of the brain sections. Although there appeared to be an area of myelin pallor (depleted myelin) that corresponded to an elevated MHC class II expression, the lesion was not easily identified. Positive staining in the sections of brain tissue however, was not only present, but was found in the anticipated locations. Anti-dsDNA antibody demonstration was seen on the perimeter of lesions and within blood vessels.

In addition to the positive fluorescence seen in the sections of tissue shown in Figures 48 – 51 and to a lesser extent, figures 52 - 53, are areas of bright yellow/orange fluorescence. These areas are thought likely to be autofluorescence resulting from lipofuscin. Lipofuscin is an autofluorescent pigment that accumulates in the cytoplasm of many cell types including those of the CNS [Schnell *et al*. (1999)]. It is composed of lipid-containing residues of lysosomal digestion in addition to sugars and metals such as mercury, aluminium, iron, copper and zinc and is thought to result from the oxidation of unsaturated fatty acids [Double *et al*. (2008)]. The presence of lipofuscin can indicate that there has been

damage to the membrane, mitochondria and/or lysosomes [Double *et al.* (2008)]. Although its presence is common in the aging animal it is also associated with diseases such as Alzheimer's, Parkinson's and amyotropic lateral sclerosis [Allaire *et al.* (2002)]. Within the sections examined, such fluorescence was only observed in the vicinity of pathological changes i.e. around active lesions and within perivascular cuffing regions. Whether such damage as indictated by lipofuscin is a by-product of the disease process, due to normal aging or directly involved in the pathogenicity of the disease is unknown. One comment to make with regard to this hypothesis is that the proposed areas of lipofuscin are largely based on H & E staining (figures 49, 51 and 53). Lipofuscin fluoresces yellow when excited at a wavelength of approximately 350 nm [Mochizuki *et al.* (1995) and Goldgefter *et al*. (1980)], figures 48, 50 and 52 are under red light of the wavelength of 650 nm and as such further investigation is required before these areas of staining can be concluded as lipofuscin.

The two different locations of anti-dsDNA antibodies raise the question of different pathogenic roles, or possibly pathogenicity being dependent on location. We know that the extent of perivascular cuffing is closely related to clinical signs and that within these areas there is a high incidence of IgG. In SLE it is thought that anti-dsDNA antibodies cause damage to blood vessels due to the deposition of immune complexes and fixation of complement leading to necrosis. It is possible that this is also true in MS, but as this is occurring in the central nervous system these seemingly small effects can cause significant disability. The research in this project

supports this as anti-dsDNA antibodies and C3 were found to present in and around vessels. In the EAE Lewis rat model it is likely that that this is the primary cause of disability and this may also be true in the early stages of MS. If this is the case then SLE research into the deposition of immune complexes could be applied to MS research.

The potential pathogenicity of anti-dsDNA antibodies on the leading edge of lesions is more difficult to explain. What appears to be clear is that they are only present on the edge of active lesions, which suggests some involvement with lesion development; whether or not this involvement is pathological or not remains to be determined. It is likely that their presence around lesions is likely to have a different role than that when they are found in and around blood vessels. DNA antibodies could be binding to oligodendrocytes which are trying to remyelinate the lesion. SLE studies in mice have found that anti-dsDNA antibodies can attach to the glomerular basement and cause damage [Han (1998)] and such antibodies from SLE patients have been found to bind to the surface of ogliodendroctes [Williamson *et al* (2001)]. Anti-dsDNA antibodies could be bordering lesions due to 'harmless' attachment to ogliodendrocytes or binding could be having a pathological effect on such cells eg inducing apoptosis or fixing complement, therefore potentially increasing the lesion area. Alternatively anti-dsDNA antibodies could be picking up apoptic cells that are present around the leading edge of the lesion.

When the positive staining of human tissue is compared to that obtained from animal tissue a similar pattern can be seen. Figure 34, a section of spinal cord from a Lewis rat sampled at peak EAE is a particularly good example of this. This section shows intermittent areas of fluorescence creating an oval shape ('lesion') very similar to that seen in figure 48 (human brain section). This correlation suggests that anti-dsDNA antibodies are present within the CNS of EAE Lewis rats and based on the staining pattern seen is likely to have a comparable role in disease pathology. It is also reasonable to hypothesise that in a relapse/remitting model or chronic model of EAE such findings would be more pronounced. To fully confirm this, a vast number of sections of CNS tissue would need to be stained and examined as the locations of lesions are not easily found.

Throughout this project it is recognised that the inclusion of controls for some aspects of the work performed was far from adequate. Although this thesis is lacking in the relevant photographs of control sections from the EAE Lewis rat, used to profile the inflammatory phase of EAE, this phase of the study did include adequate controls. Rather than photographic representation of such sections, the data has been presented graphically and textually. A number of control sections were included during the DNA probe investigations, but insufficient photographs were taken and in addition such sections were also not adequately, if at all, described. For the human tissue, controls were very limited and this was largely due to

the restricted number of sections available. Future work including sufficient controls is needed in order to substantiate these initial findings.

This research has clearly profiled the inflammatory phase of EAE in the Lewis rat and confirmed its similarities with MS. A novel and relatively simple method for the detection of anti-dsDNA antibodies *in situ* has been developed and by using this method the presence of anti-dsDNA antibodies have been found to reside in two different locations; within vessels and on the outer edge of active lesions, the latter being reported by Williamson *et al.* (2001).

The implication of this research with regard to anti-dsDNA antibodies potentially having similar pathogenicity to that seen in SLE, may be in the way in which researchers think about new therapies for multiple sclerosis. Currently the standard treatment for MS is to minimise pain, reduce the number of relapses and slow the disease progression [Andersson & Goodkin (1996)]. Treatments typically involve the use of glucocorticosteriods and corticotrophin to reduce the intensity and duration of disability associated with acute relapse [Andersson & Goodkin (1996)]. Diazepam and baclofen may be used to reduce spasticity [Andersson & Goodkin (1996)]. Amantidine hydrochloride and pemoline may be used to counteract fatigue and pain may be controlled by the use of non-steroidal anti-inflammatory agents and tricyclic anti-depressants [Andersson & Goodkin (1996)]. More recently the use of drugs targeted towards cytokines has been used. For example, copaxone, a MHC class II

modulator that has the ability to lower IL-2 levels and is thought to have a protective effect against demyelination [Ge *et al*. (2000)] and interferons (interferon beta-1a) such as Avonex and Rebif [Charatan (2000)] have be used successfully to treat symptoms of MS. Natalizumab, a monoclonal antibody which has immunosuppressant properties has also been used in MS treatment [Kappos et al. (2007)]. None of these treatments directly target anti-dsDNA antibodies. It is possible and something being considered by some researches that SLE treatments which target anti-dsDNA antibodies may also be affective in MS. Examples of drugs used in SLE which may be beneficial to MS patients by targeting anti-dsDNA antibody secretion are rituximab, LJP394 and IVIg.

Rituximab is used in the treatment of SLE. It is an antibody directed against CD20 which is expressed on B cells [Sah and Liu (2008)]. By eliminating B cells this can prevent the generation and expansion of autoreactive antibody secreting cells [Sah and Liu (2008)]. This treatment has been found to be successful in SLE patients and in some cases has been shown to decrease anti-dsDNA antibody titres. Rituximab is being trialled in MS patients and so far the results have shown promise as a treatment for relapsing/remitting MS [Hauser *et al*. (2008)]

LJP394 is a complex of four oligonucleotides which have a strong avidity for anti-dsDNA antibodies [Sah and Liu (2008)]. It has been found to decrease anti-dsDNA antibody levels, decrease the number of renal flares in SLE patients and reduced the need for corticosteroid treatment. It has

also been found to increase the time between flares [Sah and Liu (2008)]. No information could be found as to whether LJP394 has been trialled in MS patients.

Intravenous immunoglobulins are used to eliminate individual IgGs in direct proportion to their concentration. This treatment has been found to be successful in SLE and MS patients alike and is often administered when corticosteroid treatment has failed. A drawback of this treatment is that it has a limited duration in its action against pathogenic immunoglobulins [Sah and Liu (2008)].

4.1 Future work

In order to investigate the findings in the project further brain and spinal cord sections containing active lesions from deceased MS patients are required. Ideally these sections should be matched for age, sex and type of MS. This is not an easy task for many reasons. The MS tissue bank has a limited number of donations. It is unlikely that tissue is available from people who were in the early stages of the disease or having the relapsing-remitting type of MS at the time of death. The majority of tissue donations are from people suffering from chronic progressive MS, which in some way has contributed to their death. The time involved in selecting sections on the basis of active lesion presence is considerable. Tissue from patients living with MS is not an option, so correlations between disease severity/disability and histopathology can not be easily made. In view of these limitations, the availability of an animal model of relapsing-remitting and chronic progressive EAE for which demyelination and the

presence of anti-dsDNA antibodies are characteristics would enable researchers to investigate the locational pathogenicity of anti-dsDNA antibodies in more detail.

The results from this project have shown that it may be possible to detect anti-dsDNA antibodies in EAE models. However, a more thorough investigation using a much greater number of controls is required before this finding is conclusive. The presence of anti-DNA antibodies in CSF is worthy of further investigation, in particular with regard to obtaining a greater number of samples. Due to the limited volumes of CSF that can be obtained from rodents, this work would be best performed using a rat model of EAE. Additional immunohistology is required in order to confirm the positive fluorescence seen using both DAPI and Alexa Fluor/DNA probes. Dual staining could be used to identify the isotype of the antibodies. If they are found to be of the IgM class then it is likely that their role is not pathogenic, if they are of the IgG then this would put them back in the picture as potential pathogenic agents in this disease. Pharmacological studies using drugs known to target anti-dsDNA antibodies could be used to further investigate the potential of appropriate SLE drugs to modify the progression and severity of EAE and ultimately MS.

5 CHAPTER 5: CONCLUSION

This research has clearly profiled the inflammatory phase of EAE and confirmed its similarities with MS. A novel and relatively simple method for the detection of anti-dsDNA antibodies *in situ* has been developed and by using this method of anti-dsDNA antibody visualisation the results obtained have been found to support those reported by Williamson *et al.* (2001). Finally and most importantly, anti-dsDNA antibodies have been found in two different locations, which opens up the possibility that pathogenicity may be location dependent.

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