THE PATHOGENESIS AND IMMUNOREGULATION OF THE INFLAMMATORY RESPONSE IN THE CENTRAL NERVOUS SYSTEM

by

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A thesis submitted for the degree of

Doctor of Philosophy at the Australian National University

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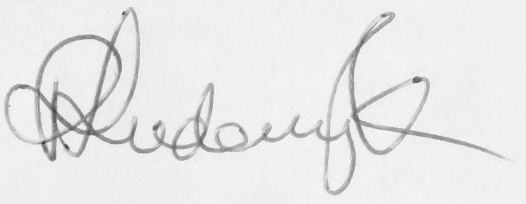
27 July 1992

STATEMENT

I certify that, except where stated the work described in this thesis is my own and has not previously been submitted for a degree at this or any other university.



Patricia Anne Ludowyk

27 July 1992

DEDICATION

I wish to dedicate this thesis to

my mother

Mercia Patricia McShane

a beautiful, talented, creative woman

who inspired me to set high standards

in all aspects of my life

and to my children

David, Sriane and Simon

for they make everything worthwhile.

ACKNOWLEDGEMENTS

I am grateful to my primary supervisor, Dr. Chris Parish, for his guidance, encouragement, enthusiasm and impressive ability to give me confidence when things weren't going as perfectly as I'd expected, to Dr. Hilary Warren for being an excellent role model and for her helpful discussions and constructive criticism when reviewing my thesis, to Dr. David Willenborg for his introduction to EAE.

I would also like to thank:

Paul Sjolema and Jenny Robinson for their assistance with the antigen-specific cell lines;

Dr. Genevieve Bennett and Leslie Maxwell (Pathology Section, Woden Valley Hospital) for their advice in interpreting histological data;

the Photographic Section, JCSMR (Stuart Butterworth, Karen Edwards, Marc Fleming and Julie Macklin) for their professional advice and help in the preparation of micrographs and figures for my seminars, publications and thesis;

the JCSMR, the Lions Club of Canberra and the Australian MS Society for providing travel funds for two overseas conferences.

PUBLICATIONS

The work contained in this thesis has been published in the following papers:

Ludowyk, P.A., Willenborg, D.O. and Parish, C.R. (1992)

Selective localisation of neuro-specific T lymphocytes in the central nervous system.

Journal of Neuroimmunology 37: 237-250

CONFERENCE PUBLICATIONS

PA Rodgers Ludowyk, DO Willenborg and CR Parish

In Vivo localisation of labelled lymphocytes in a rat model of acute experimental autoimmune encephalomyelitis.

National Multiple Sclerosis Society Scientific Meeting, Melbourne, 1989

International Symposium on Cell-to-Cell Interactions, Basel, 1990

5th International Conference of the Inflammation Research Association, Pennsylvania, 1990.

ABSTRACT

Experimental Autoimmune Encephalomyelitis (EAE), a major applied model for Multiple Sclerosis (MS) research, is also an excellent model for studying interactions between the immune system and the central nervous system (CNS). The specific aims of this study were to ascertain how T lymphocytes (T cells), which are normally found in low numbers in the CNS, localise during the inflammatory response and as MS and EAE are both age-dependent diseases, to establish the clinical, histopathological and immunoregulatory changes which occur in aged animals suffering from EAE.

In Chapter 3, using EAE in the rat as a model of CNS inflammation, activated and quiescent T cells with different antigen specificities were labelled with the fluorescent dye Hoechst 33342 and tested by fluorescence microscopy for their ability to accumulate in different regions of the spinal cord and in other organs at varying times post inoculation. With this highly sensitive assay it was found that activated myelin basic protein (MBP)-specific T cell lines accumulated in the spinal cord (a 1000 fold increase in the lumbar/sacral region by day 4) and caused clinical signs of EAE. In contrast, interleukin-2 (IL-2) maintained (quiescent) MBP-specific T cell lines failed to accumulate in the CNS and cause disease. Activated ovalbumin (OA)-specific and purified protein derivative of tuberculin (PPD)-specific T cell lines were also found at significantly higher levels in the spinal cord than non-activated cells although they failed to accumulate to a substantial degree when injected alone. When injected with activated MBP-specific T cells the activated OA-, and PPD-specific cell lines accumulated in the spinal cord following initial accumulation of the MBP-specific cells, demonstrating that during the inflammatory process there is considerable non-specific recruitment of cells into the inflammatory site. CNS accumulation of activated MBP-specific T cell lines occurred 1-2 days later in irradiated animals than in non-irradiated recipients. This was consistent with irradiated animals also exhibiting a later onset of disease and suggests that irradiation may directly affect the endothelium in a way that makes it less adhesive. Preliminary observations also supported the view that in irradiated animals tissue repair mechanisms in the CNS are compromised. Considerable hemorrhage and the exposure of collagen, a component of the extracellular matrix, were found in irradiated recipients in this study, suggesting that the integrity of the vasculature had been affected. An important observation in this study was that astrocytic proliferation was evident in irradiated and non-irradiated animals exhibiting advanced clinical signs of EAE. These results suggest that astrocytes could be important in the development of clinical signs of EAE. In conclusion, the research described in Chapter 3 demonstrates that activated lymphocytes of any specificity enter the spinal cord, and that the neuro-antigen specific cells

accumulate there and lead to the recruitment of other cells. Non-activated cells, even those with neural antigen specificity, fail to enter the spinal cord.

Chapters 4, 5 and 6 examined in detail age-related differences in EAE severity. In Chapter 4, EAE was induced in young (2-3 month old), middle-aged (12-13 month old) and geriatric (24-26 month old) Lewis (JC) rats by active immunisation with MBP in complete Freund's adjuvant (CFA). It was found that aged Lewis (JC) rats developed a more chronic form of EAE than younger rats of the same strain. Active induction showed a slower and less vigorous response in the first instance in aged animals, but the chronicity did not resolve suggesting there is a problem in resolving the neurological effects of CNS inflammation in older animals. In contrast, no significant difference in the production of MBP-specific antibodies was found between young and aged animals. Males exhibited an increased severity and chronicity of disease compared with females of the same age. However, the sterilisation of females had no effect on the onset or severity of clinical EAE in middle-aged animals. The memory response previously shown in young animals which had received EAE effectors postnatally was also evident in aged animals 13 months after receiving the effector cells.

Studies described in Chapter 5, examined whether the clinical differences in EAE chronicity between young and middle-aged animals could be attributed to gross differences in the histopathology of the disease. In these studies, the CNS inflammatory lesions were quantified, and the degree of fibrin deposition, demyelination and astrocytic hypertrophy was compared. Inflammatory lesions became apparent before the onset of disease, increased significantly during advanced clinical signs of EAE and were still evident at day 22 in young animals despite the absence of clinical signs but were largely absent from middle-aged animals which still exhibited disease symptoms. At day 40 post inoculation inflammatory infiltrates were sparse in both young and middle-aged animals despite 29% of middle-aged animals being symptomatic. These results suggest that disease chronicity does not correlate with the number of inflammatory lesions in the CNS. No significant differences were found in the degree of fibrin deposition or demyelination between young and middle-aged or symptomatic and asymptomatic animals. However, astrocytic hypertrophy was found to correlate with disease chronicity in middle-aged animals, suggesting that astrocytic hypertrophy may play a significant role in the pathophysiology of the disease.

In Chapter 6, EAE was induced in young and middle-aged naive recipients by the adoptive transfer of lymphocytes from actively immunised young donors. It was found that middle-aged recipients developed more severe disease than young recipients. Based on these observations it was concluded that disease chronicity in middle-aged animals is a property of the CNS milieu rather than a property of an ageing immune system. In parallel studies the naturally occurring pro-inflammatory mediators tumor

necrosis factor (TNF) and nitric oxide and anti-inflammatory mediators corticosterone and prostaglandin E (PGE) were examined in young and middle-aged animals using the actively induced model of EAE. No detectable levels of TNF-α were evident in middle-aged and young rats during the course of disease and no significant differences in PGE_2 levels between young and middle-aged animals or between symptomatic and asymptomatic animals were found. By contrast, markedly elevated corticosterone levels were found in both young and middle-aged animals with the development of clinical signs, which returned to baseline levels with the resolution of clinical symptoms. Elevated levels of reactive nitrogen intermediates (RNIs) were evident in animals immediately prior to and during the early stages of symptomatic EAE. Although, these results suggest that nitric oxide may play a role in the pathogenesis of disease, whereas corticosterone may play a role in the immunoregulation of the disease, these factors cannot explain differences in disease chronicity evident in middle-aged animals.

Based on the studies described in Chapters 4 to 6, it is proposed that the neurological deficit of chronic EAE in middle-aged animals may be caused by astrocytic hypertrophy with the resolution of this CNS response being a major factor contributing to recovery from the disease in animals of all ages.

TABLE OF CONTENTS

ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
BBB	blood brain barrier
B cell	B lymphocyte
BSA	bovine serum albumin
CFA	complete Freund's adjuvant
CNS	central nervous system
ConA	concanavalin A
CREAE	chronic relapsing experimental autoimmune encephalomyelitis
CsA	cyclosporin A
CSF	cerebrospinal fluid
CT	computerised tomography
EAE	experimental autoimmune encephalomyelitis
EAN	experimental autoimmune neuritis
ELAM-1	endothelial-leukocyte adhesion molecule-1
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GFAP	glial fibrillary acidic protein
GPMBP	guinea pig myelin basic protein
H33342	fluorescent dye Hoechst 33342
HEAE	hyperacute experimental autoimmune encephalomyelitis
HEV	high endothelial venule
HLA	human leukocyte antigen
ICAM-1	intercellular adhesion molecule-1
IFN	interferon
Ig	immunoglobulin
IgG	gamma globulin
IL-1	interleukin-1
IL-2	interleukin-2
IL-3	interleukin-3
IL-6	interleukin-6
JCSMR	John Curtin School of Medical Research
JHM	murine hepatitis virus
LCM	lymphocytic choriomeningitis
LFA-1	lymphocyte function associated antigen-1

LPS	lipopolysaccharide
MAG	myelin-associated glycoprotein
MBP	myelin basic protein
2ME	2-mercaptoethanol
MEL-14	murine peripheral lymph node homing receptor
MHC	major histocompatibility complex
MRI	magnetic resonance imaging
MS	Multiple Sclerosis
N-CAM	neural cell adhesion molecule
OA	ovalbumin
OD	optical density
PAF	platelet activating factor
PBS	phosphate buffered saline
PGE	prostaglandin of the E series
PGs	prostaglandins
PHA	phytohaemagglutin
PLP	bovine proteolipid apoprotein
PPD	purified protein derivative of Tuberculin
RIA	radioimmunoassay
RNI	reactive nitrogen intermediates
RPM	revolutions per minute
RT	room temperature
SLE	systemic lupus erythematosus
SPS	sulphated polysaccharide
T cells	T lymphocytes
TNF	tumor necrosis factor
t-PA	tissue plasminogen activator
tween 20	polyoxythlylene sorbitan

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Certain neurological diseases, which are characterised by inflammation and demyelination of the central nervous system (CNS), are a cause of morbidity and mortality in humans. Often, these disorders are linked etiologically by either a proven or suspected viral infection and pathologically by an inflammatory process resulting in demyelination. Multiple Sclerosis (MS) is one such disease, and is a disorder in which the mechanisms causing disease are not fully understood. It is a degenerative disease occurring when the body's immune system attacks the CNS. In this study, I will discuss the possibility that MS is an autoimmune disease resulting from a dysfunction in immunoregulation, possibly caused by a viral infection occuring during adolescence or early adulthood, which can subsequently be activated by age-related vascular and immune changes. These changes possibly cause myelin autoreactive cells, which are a direct result of the viral infection, to enter the CNS and cause inflammation and demyelination. Experimental Autoimmune Encephalomyelitis (EAE), a major applied model for MS research, is an excellent model to study the link between the immune and central nervous systems. The study of the principles of the inflammatory process within the CNS, particularly how the autoreactive T lymphocytes (T cells) can enter the CNS and cause inflammation, may lead to pharmacologic or immunoregulatory ways of manipulating the inflammatory process.

The purpose of this chapter is to:

(a) introduce the functional anatomy of the CNS and discuss the implications this has on neuropathology;

(b) discuss the relationship between the immune and central nervous systems, considering how T cells which are normally found in low numbers in the CNS, localise during the inflammatory process;

(c) consider immediate and persistent neurological damage caused by viral infection of the CNS;

(d) summarise the clinical, pathological and immunological features of the human inflammatory disorder MS; and

(e) characterise EAE as an experimental model considering the relative role inflammatory cells play in disease manifestation.

1.2 Functional anatomy of the central nervous system

1.2.1 *Development of Neural and Glial Cells*

There are basically two distinct types of cells in the central nervous system: the nerve cells and the neuroglia. Both of these cell types are derived from the epithelial cells of the ventricular zone (Schacher, 1985). Following mitosis, these cells migrate to their final destinations before undergoing differentiation (Altman, 1966). The majority of the neuronal population loses the capacity to divide prior to their migration (Giotta and Cohn, 1982). In rodents, the formation of neurons and their synapses is almost complete at birth (Altman, 1972). Although neurones are predominantly postmitotic, proliferating glia are evident throughout the brain. The two major classes of glial cells are the macroglia (astrocytes, oligodendrocytes and ependymal cells), and microglia (phagocytic cells that are mobilised during injury, infection, or disease) (Kandel, 1985). In the rat, the bulk of astrocytes are formed by the end of the third week postnatum (Raff et al., 1983; Miller and Raff, 1984) while the majority of oligodendrocytes are formed between the ages of 3 weeks and 6 months (Hertz et al., 1982). However, glial cells do not usually divide in the adult nervous system other than in response to injury or trauma (Adams, 1977; Prineas, 1975). Consequently, in the CNS of the mature animal, there is a relatively stable population of cells which are limited in their ability to regenerate.

1.2.2 *Functional role of astrocytes*

Astrocytes are classically divided into two subclasses: fibrous and protoplasmic. Fibrous astrocytes abound in glial filaments and are found in areas of the CNS containing a predominance of axons. Protoplasmic astrocytes have shorter, stouter processes that contain fewer filaments, and are associated with nerve cell bodies, dendrites and particularly synapses, which they characteristically envelop (Kandel and Schwartz, 1985). Glial fibrillary acidic protein (GFAP), an intracellular marker for astrocytes, is a major constituent of gliofilaments and antisera to the protein show intense immunohistochemical staining of the cell bodies and processes of astrocytes (Bignami et al., 1972; Raff et al., 1979). The functional role of astrocytes in cell-cell interactions in the CNS has remained largely obscure though they are thought to serve the functions set out in Table 1.1.

Table 1.1: The functional roles attributed to astrocytes:

1. **Supporting elements:** Astrocytes give firmness and structure to the brain. Their processes surround capillaries and synapses and form a continuous subpial and subependymal layer (Peters et al 1976). By wrapping areas of synaptic contact and presynaptic boutons on axons with their processes they segregate and sometimes insulate groups of neurons from each other (Miller et al, 1986). Radial astroglia in the developing brain have been proposed to act as guides for migrating neurons in the cerebellar and cerebral cortices (Schachner et al, 1982), and possibly direct the outgrowth of axons (Kandel and Schwartz, 1985).

2. **Nutrition:** Astrocyte processes have end-feet that contact both blood capillaries and neurons (Kandel and Schwartz, 1985), therefore possibly providing control over which constituents of the blood reach the neuronal surface.

3. **Scavengers:** Astrocytes remove debris and help seal-off damaged brain tissue after neuronal death or injury (Kandel and Schwartz, 1985). Several studies suggest that astrocytes proliferate in response to CNS trauma and engage in phagocytosis (Noske et al, 1982; Kandel and Schwartz, 1985; Eng, 1985) especially in those circumstances where the more active conventional phagocytes are not present in sufficient numbers (Noske et al, 1982).

4. **Scar tissue formation:** The astrocyte response to injury of the CNS is a process called reactive gliosis. Scars are formed by astrocytes extending numerous processes that become larger and have a substantial increase in glial filaments (Maxwell and Kruger, 1965; Eng, 1985).

5. **Proliferation around damaged neurons:** Astrocytes have been observed displacing presynaptic terminals along the proximal dendrites and cell bodies of axotomized motor neurons. This encroachment of astrocytes results in the damaged neurons receiving reduced synaptic input, with the evoked excitatory presynaptic potentials being smaller in amplitude (Kandel and Schwartz, 1985; Liuzzi and Lasek, 1987;).

6. **Regulation of K+ concentration:** An established electrical property for astrocytes is their large negative membrane potentials, which are predominantly K+ diffusion potentials (Kimelberg, 1983). By taking up the excess extracellular K+, astrocytes are thought to buffer the extracellular K+ concentration, protecting the membrane potential of neurons from the depolarisation that might result if K+ accumulated after repeated neuronal firing (Henn et al, 1972; Hertz, 1978; Kandel and Schwartz, 1985).

7. **Neurotransmitter uptake:** Astrocytes that surround the synaptic region, have a high affinity for certain neurotransmitters such as gamma aminobutyric acid and serotonin (Bowman and Kimelberg, 1984; Kandel and Schwartz, 1985; Kimelberg and Katz, 1985).

8. **Interaction with hormones and cytokines:** Astrocytes synthesise interleukin-3 like substances (Frei et al., 1985) and are targets for immune system cytokines and hormones (Fierz et al., 1985; Lindholm et al., 1987; Vijayan and Cotman, 1987).

1.2.3 *Age-related changes and astrocytes*

As astrocytes serve many important functions, the study of these cells is critical when assessing age-related changes in the CNS. It has been hypothesised that age-associated changes in neurochemistry reflect an underlying change in the cellular composition of the brain with an increase in astrocyte size and number and a decrease in the number of neurons (Finch and Morgan, 1990; Goss et al., 1991). Goss et al. (1991) found, as a function of age, there were increased levels of RNA for GFAP as measured by gel-blot analysis and by a solution hybridisation assay in the mouse cerebral cortex, hippocampus and cerebellum. O'Callaghan and Miller (1991), similarly found an age-related increase in GFAP in rat brains. An increase in GFAP may reflect astrocytes undergoing reactive gliosis, a process by which astrocytes increase in size and the number of processes become larger and more numerous (de Villis et al., 1986; Eng, 1985). This hypertrophy may be a reaction to the degeneration of neighbouring synapses, neurites or entire neurons (Adams and Jones, 1985; Geinisman et al., 1978; Landfield et al., 1977).

1.2.4 *Neuronal damage*

As most neurones in the adult mammalian CNS are postmitotic, they will not regenerate after physical injury as would happen elsewhere in the body and functional recovery will depend on the site of injury. These degenerative changes will occur not only in the damaged neurones but also in the neurones that receive synapses from the damaged neurones. Termed "transneuronal degeneration", the degree of atrophy is related to the reduction in total input caused by the lesion. It is postulated that neurones require a certain amount of stimulation to survive, and in addition there is possibly a trophic substance released by synaptic terminals which is necessary for their survival (Kelly, 1985). Whatever the cause, these transneuronal changes, which may be anterograde (if the affected cell receives synapses from the injured neurone) or retrograde (if the affected cell makes synapses on the injured neurone), explain why a lesion at one site in the CNS can have effects on sites distant to the lesion depending on the connections the lesion interrupts. Consequently, neuronal damage can result in long-lasting alterations in the structure and function of the affected areas.

Neuronal damage activates repair mechanisms in the CNS and the axon and myelin sheath undergo rapid local degeneration at the sites of lesions. As lesions can disrupt blood vessels, macrophages are able to enter from the general circulation and phagocytose axonal debris. Astrocytes and microglial cells proliferate and also act as phagocytes. The proliferation of fibrous astrocytes leads to the formation of a glial scar which can block the restoration of severed synaptic connections. The term "sclerosis" refers to the hard scar of astrocytes that replaces phagocytosed neuronal debris and is often used to describe diseased states such as MS. Thus, repair mechanisms activated in

the CNS by neuronal injury can be both advantageous (phagocytosis) and detrimental (blocked regeneration) (Kelly, 1985).

1.2.5 *Myelin formation*

Large neuronal axons are surrounded by the fatty insulating sheath called myelin. This insulation of axons is essential for the high-speed conduction of action potentials as the myelin sheath shields against the capacitance and conductance of the internode (Morrell and Norton, 1980). The myelin sheath is interrupted at regular intervals at nodes of Ranvier (Fig. 1.1). In myelinated axons, the action potential propagates discontinuously, in saltatory jumps from one node of Ranvier to the next, which is a faster process than the continuous propagation of unmyelinated axons (Rowland, 1985). The myelin sheath is formed by oligodendrocytes in the CNS (Fig. 1.2) and Schwann cells in the peripheral nervous system (PNS) (Peters and Vaughn, 1970). The oligodendrocyte is capable of producing many internodes of myelin (Bunge, 1968) and in the rat optic nerve 30-50 myelinated internodes are formed by a single oligodendrocyte (Peters and Proskauer, 1969). Despite different cellular origins, central and peripheral myelin share the same fundamental organisation as a compacted spiral of non-neuronal membranes (Fig. 1.3). Biochemical analysis shows that myelin has a composition similar to that of plasma membranes, consisting of 70% lipid and 30% protein, with a high concentration of cholesterol and phospholipid (Table 1.2) (Schwartz, 1985).

Table 1.2: Composition of CNS rat myelin

	% dry weight (approx.)
Cholesterol	27.7
Galactolipids	27.5
Phosphoglycerides	43.1
Sphingomyelin	7.9
Protein	
proteolipid protein	20.0
myelin basic protein	10.0

1.2.6 *Demyelination*

Demyelinating diseases are the most common causes of damage to the corticospinal system and the principle disabling lesions in MS are in the corticospinal tracts and cerebellar pathways (Traugott and Raine, 1984). Corticospinal axons extend from the cerebral cortex through the brainstem to the spinal cord, and can be damaged by lesions at any one of these locations. Weakness can be accounted for by either lesions in the upper motor neurones (pathways in the cerebral cortex, brainstem or spinal cord),

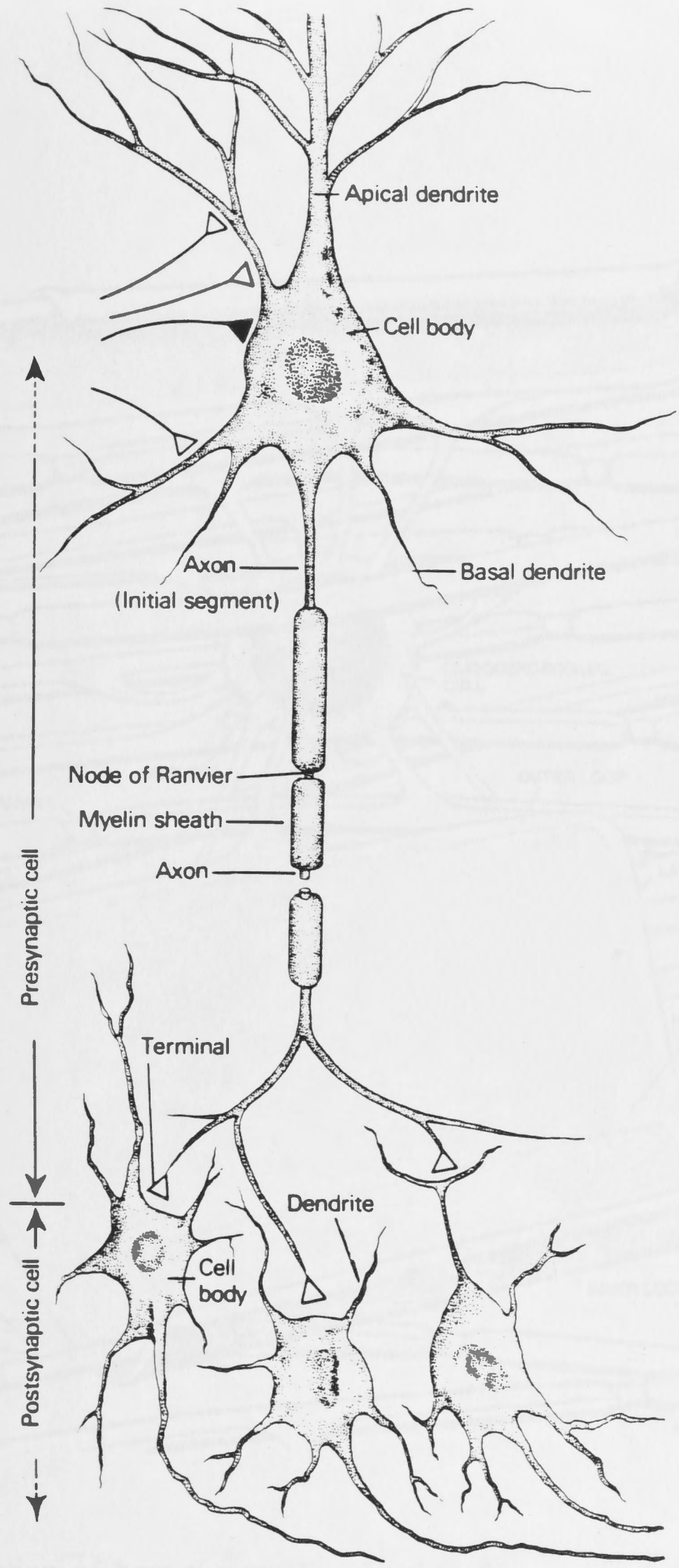


Fig. 1.1: Typical neuron illustrating the major regions. Many axons are insulated by the myelin sheath, which are interrupted at the nodes of Ranvier. As there are many points of contact from other nerve cells, the excitory presynaptic terminals are shown as white triangles and the inhibitory terminals as black triangles (Kandel and Schwartz, 1985).

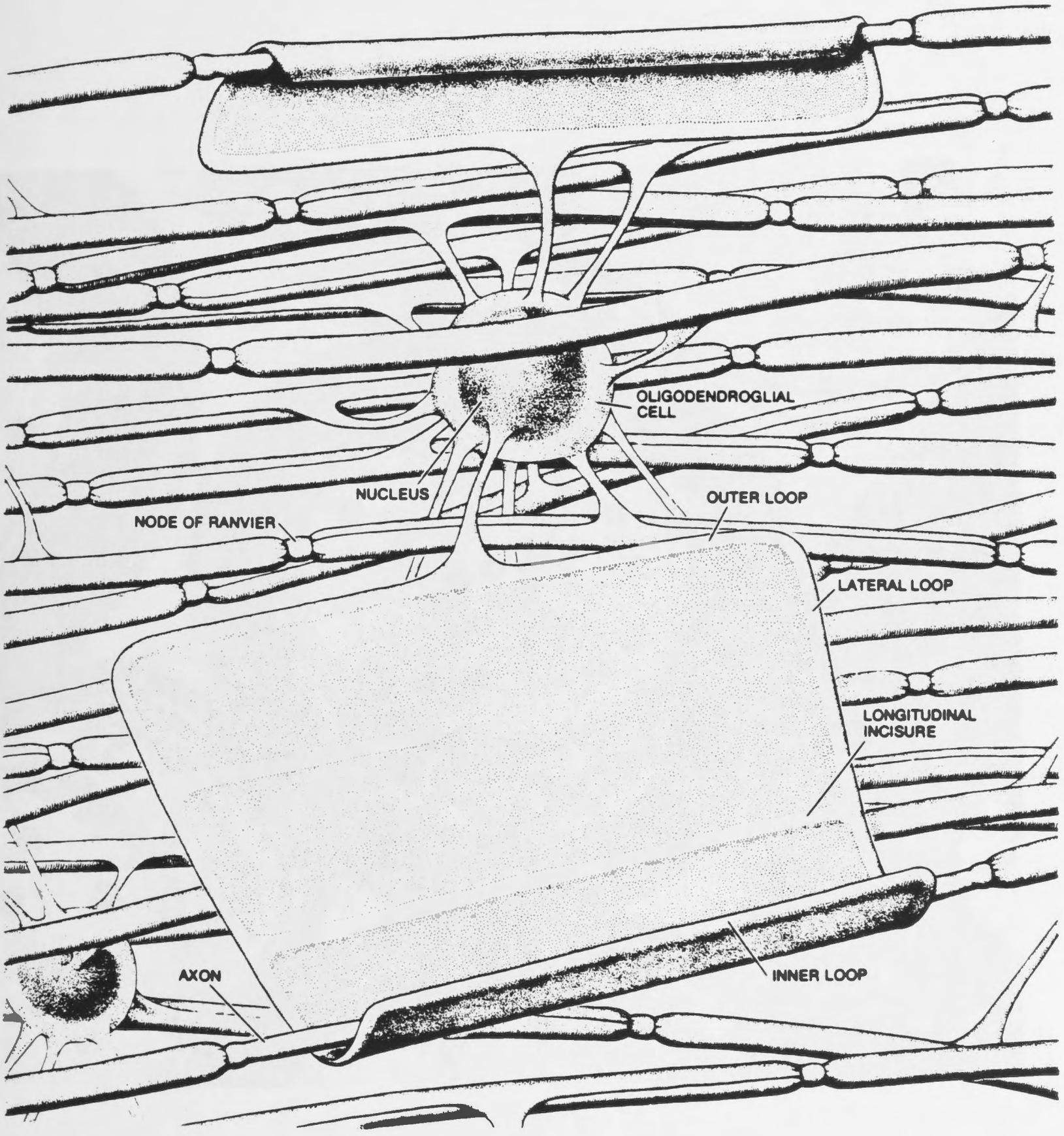


Fig. 1.2: Illustration of how the myelin sheaths of many axons are formed by an oligodendrocyte in the CNS (Morrell,1984).

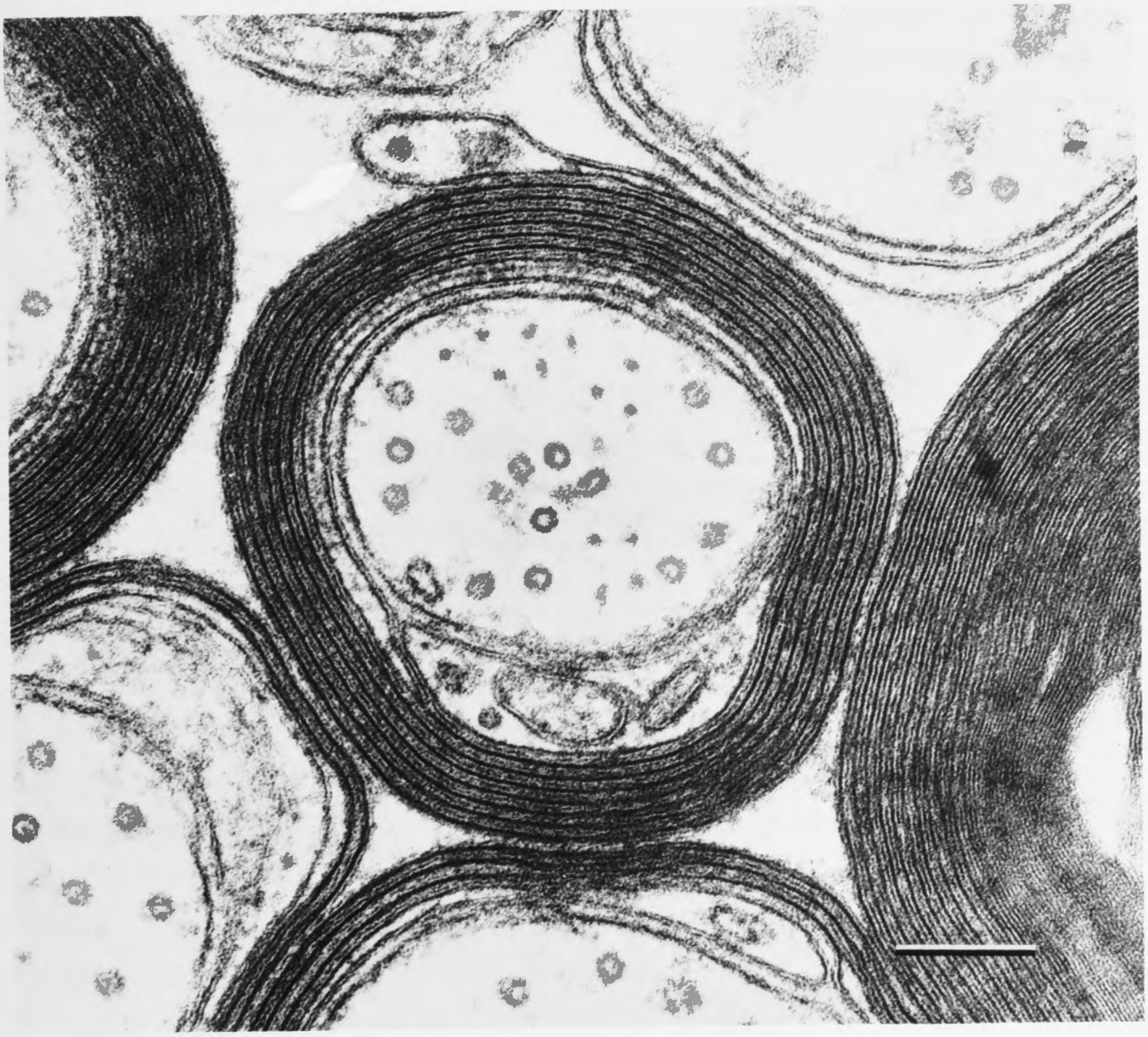


Fig. 1.3: Typical transverse section of myelin sheaths in the mature central nervous system of a dog illustrates how the myelin sheath is a compacted spiral of non-neuronal membranes (Morrell,1984).

lower motor neurones (motor neurones in the ventral horns) or the neuromuscular junction or the muscle (Bannister, 1985). The most caudal section of the CNS is the spinal cord which receives information from the skin, joints and muscles in the trunk and limbs, and issues the final commands for movement. Consequently, weakness or flaccidity in the limbs can be related to specific lesion(s) in the CNS.

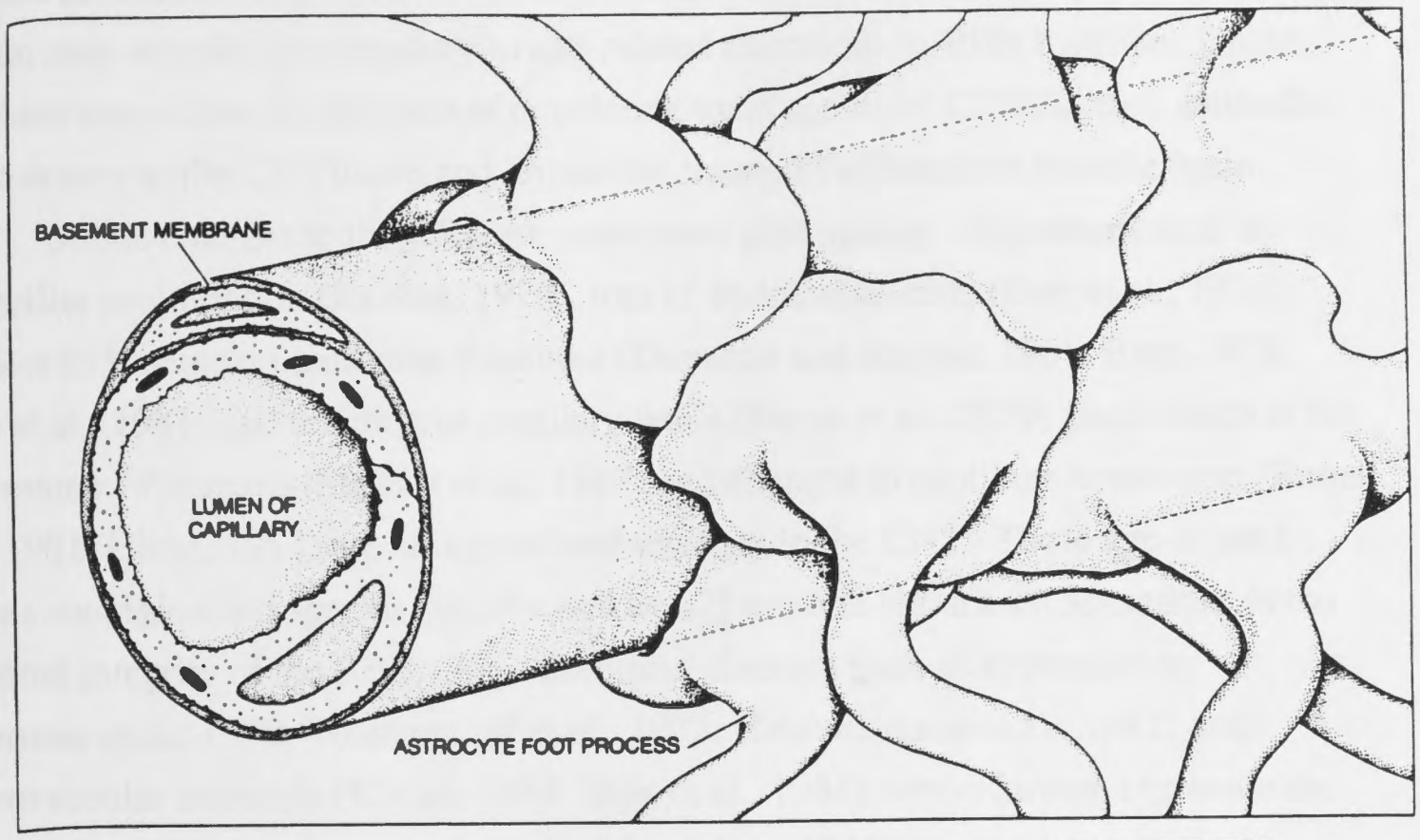
1.2.7 *The Blood Brain Barrier*

The blood brain barrier (BBB) is a system of permeability barriers which preserve homeostasis in the CNS by facilitating the entry of necessary metabolites and, blocking entry or facilitating removal of unnecessary metabolites and toxins (Fishman, 1975). The brain must be kept rigorously isolated from fluctuations in the blood composition of hormones, amino acids and ion levels, as these are capable of influencing the firing of the nerve cells. The ability of molecules to exchange across the BBB depends on their size and physical characteristics and also on the presence of specialized transport mechanisms for some substances. For instance, small molecules like sucrose enter at a relatively rapid rate; high lipid solubility of compounds enhances transport (Banks and Kastin, 1985); and nutrients (Crone, 1965; Cornford and Cornford, 1986), hormones (Banks et al., 1985) and precursors of neurotransmitters (Cornford and Oldendorf, 1975; Cornford et al., 1978) are able to cross the BBB due to specialized transport mechanisms.

The barrier function is achieved because the brain capillaries differ from vessels in other organs in that they have tight junctions between the endothelial cells. Ultrastructurally, tight junctions are where the outer leaflets of adjoining cells merge and become physically joined (Goldstein and Betz, 1986). In addition, the capillaries are surrounded by and encased in the glial foot processes of astrocytes (Rowland, 1985) (Fig. 1.4). These "foot processes" are not sealed by intercellular tight junctions and are therefore not thought to serve a barrier function. However, astrocytes may be important in maintaining the differentiation of the CNS endothelial cell (Schmidley and Maas, 1990). The basement membrane of the CNS vessels is similar to the basement membrane of other regions of the body though the structure is more compact (Schmidley and Maas, 1990). The structural characteristics of the CNS vessels consequently ensure the preservation of homeostasis within the CNS.

1.2.8 *Age related changes to the BBB*

Ageing is associated with a variety of degenerative diseases involving the CNS. The degree of CNS function deterioration is variable and probably dependent on genetic, constitutional and nutritional factors (Goodwin et al., 1983; Albalan, 1984), as well as the presence of environmental toxins (Michaelson and Bradbury, 1982; Crapper et al., 1982;



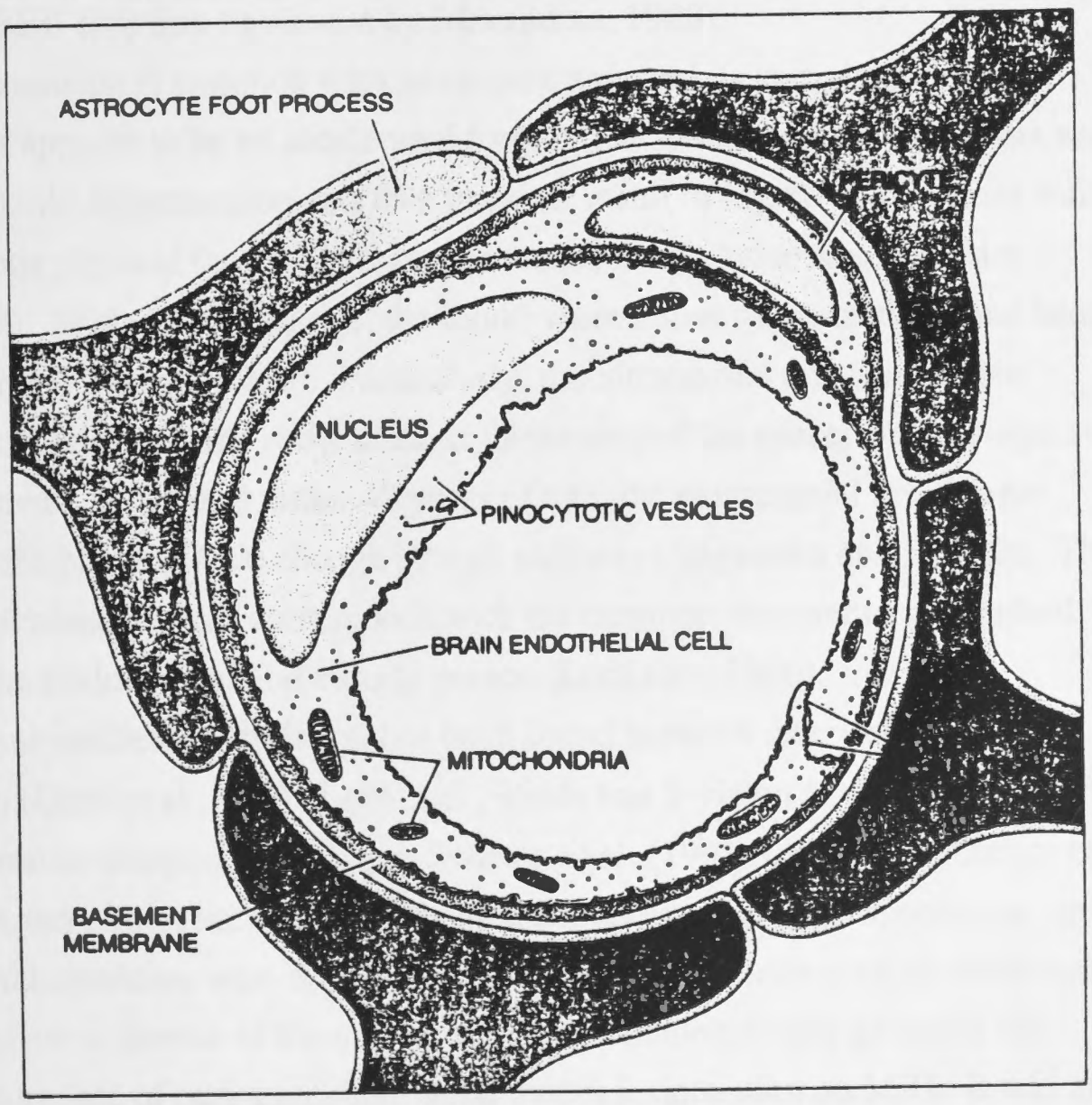


Fig. 1.4: The BBB is achieved because the endothelial cells have tight junctions and the capillary is almost completely ensheathed by astrocytic foot processes. The basement membrane supports the endothelium, enabling it to maintain its tubular form (Goldstein and Betz, 1986)

Banks and Kastin, 1983). As the BBB is a major determinant in the delivery of nutrients or environmental toxins to the CNS, it is of extreme importance to know what happens when the functional integrity of the BBB declines. The age-related decline in CNS function may actually be secondary to age-related alterations in BBB function. These alterations may allow the entrance of circulating toxic agents or CNS directed antibodies to gain access to the CNS tissue and impair the transport of nutrients into the brain.

Subtle changes to the BBB are associated with ageing. Alterations such as gliofibrillar proliferation (Ravens, 1978), loss of endothelial cells (Barr et al., 1978) increases in basement membrane thickness (Donahue and Pappas, 1961; Barr, 1978; Burns et al., 1981) and thinning of capillary walls (Burns et al., 1979) particularly in the white matter of humans (Stewart et al., 1987) and changes in capillary lumen size (Burns et al., 1981; Hicks, 1983) are all age-related changes in the CNS. These age-related changes are region and species-specific and would result in significant alterations in the functional integrity of the BBB. Age associated diseases such as hypertension (Johansson et al., 1970; Westergaard et al., 1977; Hatzinikolaou et al., 1981) and cerebrovascular ischemia (Klatzo, 1983; Sage et al., 1984) would further aggravate the alterations in BBB function (reviewed by Mooradian, 1988).

Hypertension is common with advancing age, and its complication, arteriosclerosis appears to be an accelerated form of the ageing process (Ooshima and Yamori, 1980). As hypertension acts as a physical insult or injury to the vessel wall, it is the most obvious physical factor which compromises the endothelial cell barrier (Hazzard,1989). With advancing age, the blood vessels lose their elasticity and become stiff (Ooshima and Yamori, 1980). Particularly, the intracranial arteries become increasingly stiff with age and show marked thickening of the vessel walls (Nagasawa et al., 1982) whereas, in humans under 40 years of age, the extracranial arteries are relatively distensible with little change in wall stiffness (Nagasawa et al., 1982). This feature of intracranial arteries corresponds with the common occurrence of cerebral aneurysm in the middle-aged and elderly person (Locksley, 1966).

In some studies a correlation has been found between arteriosclerosis and demyelination (Goto et al., 1981; Lotz et al., 1986; van Swieten et al., 1991). Using magnetic resonance imaging (MRI), van Swieten et al. (1991) found the presence of demyelination and gliosis accompanied by arteriolosclerosis in brain specimens. In contrast, normal arterioles were always found in conjunction with normal white matter. These findings are in favour of the notion that demyelination and/or gliosis is the pathological correlate of periventricular white matter lesions seen on MRI. It was also suggested that arteriolosclerosis precedes and probably causes the demyelination and loss of axons (van Swieten et al., 1991).

There is some evidence to suggest that astrocytes undergo structural changes during ageing and disease which results in partial loss of the BBB. During cerebral infarction or severe head trauma, there is an increase in the levels of extracellular K+, which is taken up by astrocytes. The cellular oedema that developes is confined to astrocytes (Schmidley and Maas, 1990). In Reye's syndrome, a disorder which usually follows a viral infection and is characterised by encephalopathy with severe brain oedema, there is swelling of astrocytes and focal expansions of the myelin sheath (Schmidley and Maas, 1990).

Under normal conditions the BBB is assumed to impede the entry of immunoglobulins and immunocompetent cells from the peripheral circulation to the CNS (Rappoport, 1976). The diffusion of immune elements such as brain-reactive antibodies (antibodies which can react with brain antigens), which are found in increased numbers as a function of age (Nandy, 1972a; Feden et al., 1979; Baldinger and Blumenthal, 1983; Blumenthal, 1988; Blumenthal et al., 1984; reviewed by Forster and Lal, 1991), may be possible when the function of the BBB is disrupted. For instance, Ig binding to CNS neurons was demonstrated in aged mice, which had circulating brain-reactive antibodies but not in young mice where these circulating antibodies were not present (Nandy, 1972b). The age-related increase in permeability of the BBB (reviewed by Mooradian, 1988) may permit the diffussion of brain-reactive antibodies into CNS tissue (Nandy, 1975; Forster and Lal, 1991).

Brain oedema can occur in patients during infectious, vascular, metabolic, neoplastic, toxic and traumatic brain disorders, although the most common is vasogenic brain oedema (Schmidley and Maas, 1990). During vasogenic brain oedema, the disrupted tight junctions which normally allow very little protein to enter the CNS, break down and the extracellular fluid is found to be high in plasma protein (including immunoglobulins). Vasogenic oedema also shows a predilection for white matter and it is believed that the parallel bundles of myelinated axons making up the white matter offer less resistance to the flow of extracellular fluid than the densely packed neuronal and glial processes of the gray matter (Schmidley and Maas, 1990). Consequently, there is a mechanism suggesting that neuropathology can be attributed to pathologic changes to the BBB associated with the ageing process.

1.3 Link between the immune and central nervous systems

The relationship between the immune and central nervous systems is complex and despite the endeavours of many researchers is poorly understood (Ader, 1981, 1991; Leibowitz and Hughes, 1983; Behan and Spreafico, 1984). The central nervous and immune systems are physically separated as the CNS lacks an extensive lymphatic system

and is protected by the BBB which is thought to be impermeable to immunoglobulins and lymphoid cells. It has been found in many species, that under normal conditions lymphocytes are found in low numbers in the CNS (Hauser et al., 1983; Hickey et al., 1983; Booss et al., 1983; Lassmann et al., 1986; Raine and McFarlin, 1985) and it is only when disease occurs that components of the immune system become apparent (reviewed by Hickey, 1991). How their entry is achieved in the absence of gross damage to the vascular endothelium is unknown and is one of the subjects of investigation in Chapter 3 of this report. The ability of glial cells to synthesise a variety of lymphokines and cytokines within the CNS, which influence the growth and function of lymphoid cells (reviewed by Benveniste, 1988), may be important in the development of intracerebral immune responses. Two questions which need to be answered are: is the CNS an immunologically privileged site and if so, how then do lymphocytes become apparent in an organ from which they are normally excluded?

1.3.1 *Immunological surveillance of body tissues*

The generalised immunological surveillance of body tissues is achieved by the constant recirculation of lymphocytes between the bloodstream and lymphoid tissue (Gowans, 1957, 1959a, 1959b; Gowans and Knight, 1964). This process maximises exposure of a captured antigen in a particular lymphoid organ to migrating lymphocytes, only a few of which can respond to it (reviewed by Kieran et al., 1989; Yednock and Rosen, 1989). Although lymphocytes have the potential for universal migration, they display migratory preference for particular lymphoid tissues (Butcher et al., 1980; Stevens et al., 1982; Chin and Hay, 1984) and sites within those tissues (de Sousa, 1981). Extensive reviews have been published demonstrating these selective lymphocyte migratory patterns (Jalkanen et al., 1986b; Butcher, 1986; Woodruff and Clarke, 1987; Berg et al., 1989; Duijvestijn and Hamann, 1989; Hamann and Thiele, 1989; Pals et al., 1989; Yednock and Rosen, 1989).

1.3.2 *Antigen presentation in the central nervous system*

Unlike antibody, T cells cannot recognise free antigen but are dependent on antigen presentation by accessory cells. Antigens have to be seen in association with glycoproteins encoded by genes of the major histocompatibility complex (MHC) (Zinkernagel and Doherty, 1976; Doherty et al., 1977). There are two different types of MHC glycoproteins designated class I and class II found on the cell surface which are thought to target functionally different types of T cells. Helper (CD4+) T cells are targeted onto the class II MHC antigens while cytotoxic (CD8+) T cells interact with the class I MHC antigens (Steinmetz and Hood, 1983; Doherty et al., 1984).

CNS cells usually display low or virtually undetectable levels of MHC glycoproteins compared with other cells in the body (Williams et al., 1980; Ting et al., 1981; Hauser et al., 1983; Wong et al., 1984; Sobel et al., 1984; Hickey et al., 1985; Lampson and Hickey, 1986; Lassmann et al., 1986; Fontana et al., 1987) although MHC molecules play a fundamental role in the induction and regulation of immune responses. Gamma-interferon (IFN-γ), a lymphokine produced by activated T cells (Morris et al., 1982; Sethi et al., 1983) induces an increase in the expression of class I MHC on astrocytes, oligodendrocytes, microglia and neurones in rat, mice and humans (Wong et al., 1984, 1985; Suzumura et al., 1986; Hirayama et al., 1986). Class II MHC antigens may be expressed at low levels due to the local production of prostaglandins (PGs). Alternatively, the stimulus for class II MHC antigen expression may be lacking, eg. by the failure of substances such as IFN-γ to enter the brain due to the existence of the BBB (Fontana and Fierz, 1985). Class II MHC antigen expression can be enhanced by viral infection (Massa et al., 1987) or by the addition of IFN-γ (Wong et al., 1984; Fontana et al., 1984). Although, the primary function of IFN-γ in the CNS is thought to be the enhancement of class I and II MHC antigen expression on various brain cell types, IFN-γ also activates macrophage killing, enhances the function of natural killer (NK) and cytotoxic T cells, and modulates antibody production (Wong et al., 1985). Thus IFN-γ may render brain cells competent to initiate and participate in both immunoprotective and immunopathological responses in the brain.

1.3.2.1 Astrocytes Act as Immune Accessory Cells

Previous research suggests that astrocytes may form the active contact between the immune system and the brain. There is intense contact between astrocytes and brain vascular tissue at the site of the BBB (Peters et al., 1976); astrocytes are capable of expressing class II MHC molecules and presenting antigen to T cells (Fierz et al., 1985; Fontana et al., 1984, 1987; Wekerle, 1986; Wekerle et al., 1987); class II MHC antigen expression is enhanced on astrocytes by IFN-γ (Hirsch et al., 1983; Wong et al., 1984) or by virus particles of murine hepatitis virus (JHM) (Massa and Ter Meulen, 1987); this IFN-γ-induced expression is further enhanced by tumour necrosis factor-α (TNF-α) (Benveniste et al., 1989; Vidovic et al., 1990); astrocytes can be stimulated to secrete interleukin-1 (IL-1) (Fontana et al., 1982), interleukin-3 (IL-3) (Frei et al., 1985), interleukin-6 (IL-6) (Frei et al., 1989; Benveniste et al., 1990), PGs (Fontana et al., 1982) and TNF-α (Robbins et al., 1987; Lieberman et al., 1989; Chung and Benveniste, 1990); astrocytes can be stimulated to proliferate by lymphokines released by T cells (Fontana et al., 1981; Merrill et al., 1983) and it has been suggested that IL-3 released by astrocytes could act locally as a growth factor for the proliferation of microglial cells and macrophages (Benveniste, 1988).

Fontana and Fierz (1985) put forward the following hypothesis to explain the way in which astrocytes may interact with endothelial cells to determine the immune reactivity of the CNS. Initially, astrocytes could be involved in the bi-directional transport of antigens between endothelial cells (which have access to the circulation) and brain tissue. Lymphocytes passing through brain vessels would recognise the antigen on endothelial cell surfaces and interact with the endothelial cells to disrupt the endothelial tight junctions by a mechanism not yet established. This would allow T cells to enter the brain and astrocytes would then support the intracerebral T cell activation process. The capacity of astrocytes to present antigen would be enhanced by increased class II MHC expression due to T cell signals such as IFN-γ and T cell activation would be further developed. The extent of intracerebral T cell activation may be limited by prostaglandin E (PGE) which is released by activated astrocytes and can lead to inhibition of class II MHC expression (Fontana and Fierz, 1985). Thus, there is evidence of a communication between astrocytes and immune cells which is mediated by cytokines.

1.3.2.2 Microglial cells as Immune Accessory Cells

It has also been suggested that the microglial cell is the principle antigen presenting cell in the CNS, responding to stimuli ranging from trauma to degenerative diseases (Vass et al., 1986; Woodroofe et al., 1986; Hayes et al.,1987; Hickey and Kimura, 1987, 1988; Hickey et al., 1987; McGeer et al., 1987,1988; Cruzner et al., 1988; Graeber et al., 1989; reviewed by Hickey, 1990). The origin of microglial cells is unknown, though they have been shown to be related to the macrophage/monocyte family (Giulian and Baker, 1985; Matsumoto et al., 1986; Baker et al., 1986; Giulian, 1987; Perry and Gordon, 1987,1988; Frei et al., 1987, 1988; Graeber et al., 1988; Giulian and Ingeman, 1988; Hickey and Kimura, 1988). Microglial cells have been shown to express MHC antigens and to synthesise immunoregulatory molecules such as IL-1, IL-6, TNF-α and PGE_2 (Giulian et al., 1986; Frei et al., 1987, 1988, 1989; Giulian, 1987; Northoff et al., 1989; Gebicke-Haerter et al., 1989) as well as secreting substances which stimulate astrocytes to become reactive (Giulian and Baker, 1985; Giulian, 1987; Graeber et al., 1988). Microglial MHC expression (classI and class II) is induced *in vivo* within 48 hours of intravenous injection of TNF and IFN-γ in rats (Male and Pryce, 1988; Steiniger and van der Meide, 1988). Microglial cells can also become phagocytic (Mori and Leblond, 1969), express gamma globulin (IgG) receptors and function as antigen presenting cells *in vitro* (Giulian et al., 1986; Giulian, 1987; Frei et al., 1987, 1988,; Graeber et al., 1988; Northoff et al., 1989).

Microglial cells are generally thought to act as scavenger cells during injury or inflammation of the CNS as they become activated, undergo rapid proliferation and migrate towards the inflammatory site. It has been proposed by Giulian et al.(1986) that

microglial cells are the first brain cells to appear in increased numbers at inflammatory sites and that the release of IL-1 from microglial cells stimulates the proliferation of nearby astrocytes. IL-1 has been demonstrated to have a stimulatory effect on astrocytes (Giulian and Lachman, 1985). As both astrocytes and microglial cells release immunoregulatory molecules as well as express class II MHC upon stimulation with IFN-γ, they could both be important immune accessory cells.

1.3.2.3 Endothelial cells as Immune Accessory Cells

The vascular endothelium may also play an important role in inflammation. The endothelial cells line the vessels and therefore provide the barrier between circulating T cells and the extravascular sites of antigen. Endothelial cells can also be induced by IFN-γ to express class II MHC (Colson et al., 1987; Markus et al., 1987) and increase the expression of cell adhesion molecules such as lymphocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1) (Dustin and Springer, 1988). Freshly isolated human endothelial cells have been found to be as effective as macrophages in antigen presentation (Burger et al., 1981). Thus, it is possible that lymphokines may increase the adhesiveness of leukocytes to endothelium at sites of inflammation.

1.3.2.4 Shared antigens on nervous system and lymphoid cells

There is considerable evidence for shared antigenic determinants between nervous system and lymphoid cells (Fuchs et al., 1980; Oger et al., 1982; Garson et al., 1982). Antigenic structures which are shared on nervous system and lymphoid cells are the IL-2 receptor (Hofman et al., 1986), CD4 antigen (Funke et al., 1987; Dewhurst et al., 1987), IFN-γ and IL-6 receptors (Benveniste et al., 1990) and class I and II MHC antigens (Wong et al., 1984; Fierz et al., 1985). The shared antigenic determinants between nervous system and lymphoid cells may be relevant in the pathogenesis of human inflammatory disorders of the CNS.

1.3.3 *T cell entry into the central nervous system*

In order to enter the CNS parenchyma, lymphocytes must first adhere to and then migrate through the CNS endothelium and the subendothelial extracellular matrix. The mechanism of this initial entry into the CNS is unknown.

1.3.3.1 Antigen specificity

Could T cell entry into the CNS be dependent on antigen specificity? If only T cells specific for CNS antigens were permitted to enter the CNS, then all the T cells in the cerebro-spinal fluid (CSF) would need to be specific to CNS antigens. This is not the

case (Clark et al., 1984). It has been found that lymphocytes which have specificities which are non-specific to the CNS are able to move from the circulation to the CNS (Wekerle et al., 1986, 1987; Hickey et al., 1989; Meyermann et al., 1986) implying that any T cell regardless of antigen specificity can gain access to the CNS. Consequently, antigen specificity is not thought to be a mechanism of initial entry of lymphocytes into the CNS though specific cell surface molecules are thought to play an important role in these interactions.

1.3.3.2 Adhesion molecules implicated in lymphocyte migration

LFA-1 and ICAM-1 interactions have also been strongly implicated in lymphocyte migration and cell-mediated immune reactions such as antigen presentation (Bierer and Burakoff, 1988; Maio et al 1989; Altmann et al 1989). LFA-1 is a member of the integrin family and is expressed virtually on all lymphocytes (Hynes, 1987). LFA-1 has been identified as a facilitator of T cell recognition and as a T cell adhesion molecule (Kishimoto et al., 1989; Shaw et al., 1986 and 1990; Shaw and Luce, 1987). Studies by Hamann et al. (1988) revealed that antibodies to LFA-1 inhibit lymphocyte attachment to specialised postcapillary venules, termed high endothelial venules (HEVs) *in vitro* and decrease the migration of normal lymphocytes into lymph nodes and Peyer's patch by 40-60% *in vivo*. It has been suggested that LFA-1 acts as an accessory molecule supporting weak interactions between cells, as cells containing high levels of murine peripheral lymph node homing receptor (MEL-14, an antigen associated with mature, recirculating, unstimulated lymphocytes) are less susceptible to inhibition by anti-LFA-1 than those with low levels (Harder and Heinz-Günther, 1988).

ICAM-1 was initially identified as a B lymphocyte (B cell) activation marker (Clark et al., 1986) and a ligand for LFA-1 (Rothlein et al., 1986; Simmons et al., 1988). ICAM-1 is expressed on many cell types (Shimizu and Shaw, 1990) and is induced on others in inflammation following exposure to lymphokines such as IL-1, TNF-α, IFN-γ and lipopolysaccharide (LPS) (Boyd et al., 1988; Dustin et al., 1988; Dustin and Springer, 1988; Marlin and Springer, 1987; Simmons et al., 1988; Rothlein et al., 1988; Staunton et al., 1988). ICAM-1 is a member of the immunoglobulin (Ig) supergene family (Wawryk et al., 1989) and shares sequence homology with N-CAM and MAG (myelin-associated glycoprotein) (Simmons et al., 1988; Staunton et al., 1988).

The expression of ICAM-1 on CNS endothelial cells and glia in sites of active inflammation could relate to their hypothesised immune functions within MS plaques (Traugott et al., 1985; Male et al., 1987; Fontana et al., 1984; Hayes et al., 1987) and in other lesions. Sobel et al. (1990) immuno-stained postmortem CNS tissues from patients with CNS inflammatory and noninflammatory conditions with antibodies to ICAM-1 and LFA-1. Their findings suggest that vascular ICAM-1 may be upregulated early and

focally in inflammatory lesions in the CNS and possibly participate in the regulation of the focal accumulation of leukocytes in CNS immune reactions through interaction with LFA-1.

1.3.3.3 The role of sulphated polysaccharides in lymphocyte migration

Recent studies have suggested that sulphated polysaccharide (SPS) recognition may also play a key role in the migration and positioning of lymphocytes within lymphoid tissues and in a range of cell-adhesion systems including neuronal cell-cell adhesion (Cole et al., 1986). Receptors for SPSs are present on a wide variety of cell types including lymphocytes (Bradbury and Parish, 1989; Parish and Snowden, 1985; Parish et al., 1984, 1988; Thurn and Underhill, 1986), macrophages (Bleiberg et al., 1983; Chong and Parish, 1986), endothelial cells (Glimelius et al., 1978; Glabe et al., 1983b) and rat sympathetic neurones (Vidovic et al., 1986). Certain SPSs, such as dextran sulfate and heparin, cause leucocytosis and inhibit lymphocyte recirculation when injected into animals (Bradfield and Born, 1974; Jansen et al., 1962; Sasaki and Suchi, 1967) whereas fucoidan inhibits lymphocyte adhesion to HEVs *in vitro* (Stoolman et al., 1987; Stoolman and Rosen, 1983). The most conclusive *in vivo* evidence for SPS involvement in lymphocyte recirculation comes from the work of Brenan and Parish (1986) which demonstrated the selective effects of SPS on entry, displacement and subsequent positioning of lymphocytes within lymphoid organs. SPSs appeared to block SPS receptors on the surface of endothelial cells and decrease lymphocyte entry. Lymphocytes were also displaced from their normal positions in the red and white pulp of the spleen by fucoidan and dextran sulfate.

SPSs are also capable of inhibiting EAE (Willenborg and Parish, 1988; Lider et al., 1989) by preventing entry of lymphocytes into sites of inflammation in the CNS. Since encephalitogenic cells when stimulated with antigen produce elevated levels of heparan sulphate endoglycosidase (Naparstek et al., 1984; Lider et al., 1989), a critical enzyme in the passage of leukocytes through the vascular extracellular matrix, it is thought that SPSs inhibit this enzyme and thus prevent development of EAE (Willenborg and Parish, 1988; Lider et al., 1989). Furthermore, although the mechanism of entry of lymphocytes into the CNS is unknown, entry is thought to be dependent upon the activation state of the lymphocytes (Naparstek et al., 1983, 1984; Meyermann et al., 1986; Wekerle et al., 1987; Hickey et al., 1989). Wekerle et al. (1986) have proposed that initial emigration of lymphocytes into the CNS is not immunologically specific and that activated T cells of any specificity can and will emigrate through CNS endothelium into the parenchyma of the CNS. They suggest that the process acts as an immune surveillance mechanism. If the activated cells entering the CNS encounter an antigen for which they have a specificity, they remain, otherwise they may die or move back out of

the CNS. Activation is thought to result in the expression of enzymes, such as heparan sulphate endoglycosidase, on the cell surface that enable these lymphocytes to migrate through vascular endothelium by degrading the vascular extracellular matrix.

1.3.4 *Immune Responses to Viruses in the CNS*

In addition to causing acute and chronic inflammatory disease, viral infections of the CNS in humans and animals have been associated with malformation, degenerative disease, demyelinating disease, neoplasms and vascular disease (Wolinsky and Johnson, 1980). This extraordinary diversity of pathological reactions can be explained by the vulnerability of CNS cells to different types of viruses. During infection, CNS cells differ enormously in their response to viral infection and range from high susceptibility to complete resistance (Johnson, 1980). For instance, poliovirus infection appears to involve only motor neurons resulting in flaccid paralysis without sensory abnormalities (Wolinsky and Johnson, 1980) whereas the JHM strain of mouse hepatitis virus causes acute demyelination in mice by the selective infection of oligodendrocytes (Lampert et al., 1973; Johnson, 1980).

The mature CNS represents an organ of extraordinarily high metabolic activity composed of a stable cell population that has only limited capacity to regenerate and little normal turnover of cells (Wolinsky and Johnson, 1980). This results in very different clinical features developing from viral infections initiated at different developmental periods. For instance, cytomegalovirus, which in adults is fairly common and usually innocuous may lead to deafness and subnormal intelligence when it infects the foetal brain. Likewise the transmission of rubella from mother to foetus in the first trimester of pregnancy, can cause mental retardation and infantile autism (Kety, 1979). Thus acute infections, although of limited duration, can have permanent effects on CNS cells.

1.3.4.1 Demyelination in viral infections of the CNS

Watanabe et al. (1983) suggested that a viral infection of glial cells could initiate an autoimmune response causing exacerbating demyelinating disease. Myelin breakdown can be accounted for by increased proteolytic activity at the edges of MS plaques (Einstein et al., 1972) which could be caused by an immune response to a non-myelin component. Wisniewski (1977) decribed this as the "bystander" effect where the expression of a viral antigen at the surface of a glial cell could stimulate an immune response with resultant release of proteolytic enzymes. Myelin has a high susceptibility to enzymic digestion and the release of the proteolytic enzymes could result in breakdown of adjacent myelin. Thieler's murine encephalomyelitis virus, an immunologically mediated, MHC influenced disease which results in demyelination (Clatch et al., 1986; Melvold et al., 1987) could be an example of this type of "bystander" demyelination. The extent of demyelination in

Thieler's disease actually correlates best with the presence of macrophages rather than the amount of virus (Lipton and Dal Canto, 1979). Demyelination caused by macrophages has also been illustrated in a variety of viral infections (Lampert et al., 1973; Dal Canto and Lipton, 1975; Lampert, 1978). The macrophages penetrate the sheaths where the myelin lamellae have dissolved or at nodes of Ranvier and insinuate themselves either within or between the myelin sheaths (Lampert, 1967). Infection of Lewis rats with a neurotropic measles virus results in subacute encephalomyelitis which is associated with a cell mediated autoimmune response to myelin basic protein (MBP) (Liebert et al., 1990). It is evident from these results that demyelination in the CNS similar to that found in MS can occur as a result of a viral infection.

1.3.4.2 Viral persistence in the CNS

As a consequence of the BBB, the CNS is partially sequestered from the immune system and under normal conditions the brain is relatively devoid of immunocompetent or phagocytic cells. Consequently, a foreign particle such as a virus in the CNS will not be phagocytozed and processed by scavenger cells as it would in other areas of the body (Wolinsky and Johnson, 1980; Johnson, 1982). High molecular weight constituents of blood such as antibody and complement are relatively excluded by the BBB (Norrby, 1978). Immunity may be provided by immunoglobulins that do transfer across the BBB or by immunoglobulins produced locally by a restricted number of antibody-producing cells harboured in the CNS (Vandvik and Norrby, 1973; Kristensson and Norrby, 1986). After infection is established, mononuclear cells appear to enter the CNS, clonal expansion of these cells is stimulated (Kristensson and Norrby, 1986) and the inflammatory response develops. As tissue destruction and inflammatory reactions proceed, these barriers to the diffusion of large molecules into and within the brain become less substantial (Wolinsky and Johnson, 1980).

The physical nature of the BBB deters virus invasion of the CNS, yet once invaded these same barriers form an impediment to clearance of infection (Wolinsky and Johnson, 1980). Viruses can persist and cause chronic disease during immune responses if latency is established, if replication and spread of the virus is restricted or if the virus is non-antigenic and fails to incite host responses (Wolinsky and Johnson, 1980). For instance, several of the herpes viruses (varicella-zoster, herpes simplex virus types 1 and 2) move centripetally along sensory axons and establish latency in the sensory ganglia (Stevens, 1978; Johnson, 1984). The viral genome is apparently either integrated into neuronal DNA or sequestered within the episome. The static cell provides a life-long repository for viral genetic information which can subsequently be reactivated to cause disease (Johnson, 1984).

1.3.4.3 Viral latency in the CNS

In subacute sclerosing panencephalitis, an uncommon complication of measles virus infection, virus appears to be adequately cleared during the initial infection. However a late disease develops and is associated with anti-viral antibody in both sera and the CNS (Aganarsdottir, 1977). The viral antigens remaining on the surface of infected cells are not fully enveloped. The virions are defective in the brain, but once established in culture are capable of replicating in permissive cells to yield infectious virus (Kipps, 1983). This defect may lead to slow cell-to-cell spread of virus and to lack of efficient clearance despite a strong immune response (Wolinsky and Johnson, 1980).

Oldstone and colleagues (1984) also found that a virus can cause injury by altering the normal functions of cells without destroying the cells. Pituitaries from lymphocytic choriomeningitis virus (LCM) infected mice were found to contain viral antigens but demonstrated no evidence of altered morphology by low power or high resolution microscopy. Despite this the animals were abnormal and it was found that synthesis of a hormone needed for growth and glucose regulation was absent. It was found that viral replication had occurred in the specialised cells making the hormone although morphology was normal (Oldstone et al, 1984).

Cross-reactivity between viral and host antigens may occur due to similarity of viral and host epitopes or alternatively, when a virus buds through the host cell membrane it is possible that host cell membrane proteins are included in the envelope of lipid-enveloped viruses. It is consequently natural to ask whether demyelinating disorders could be caused by previous viral infections. Waksman and Reingold (1986) have postulated that this autoimmune mechanism could cause MS. Autoimmune demyelination could arise through molecular mimicry (Fujinami and Oldstone, 1985; Janke et al., 1985; Oldstone, 1987; Shaw and Luce 1987) and it is plausible that demyelination may result from vascular changes causing increased BBB permeability with the resultant entry of autoreactive T cells directed against myelin, which were initially caused by a viral infection.

1.3.5 *Age related changes in the immune system*

There is a drastic decline in immune competence with age (reviewed by Gottesman and Walford, 1982). Two functional changes in the immune system which are evident with age are the decline in the immune response to exogenous stimuli and a loss of self tolerance. These changes are thought to be associated with the increased incidence of infectious and autoimmune diseases observed in the elderly (Ackerman et al., 1991).

Autoimmune diseases are characterised by the failure of the immune system to distinguish self and non-self. Taguchi et al. (1990) suggested that every organ in the

body produces tissue-specific antigens, which may be released into the blood stream and encounter the immune system. Possible explanations of why the normal immune system does not react to these antigens are that:

a) T cell clones reactive to self antigens are positively eliminated in the thymus (clonal deletion theory);

b) self-reactive clones are in a long-term state of unresponsiveness (clonal anergy theory); and

c) peripheral auto-reactive T cells are controlled by specifically organised T cells (suppressor T cell theory) (Schwartz, 1989; Taguchi et al., 1990).

The appearance of cells with non-self characteristics could be accounted for by the error-autoimmune theory where it is suggested that cells can be incorrectly specified (Blumenthal and Berns, 1964; Blumenthal and Probstein, 1968; reviewed by Blumenthal, 1976). The immune system normally provides an adaptive mechanism which removes cells which are specified incorrectly but with ageing this function is decreased and cells acquiring "non-self" characteristics may evoke an immune response.

The decline in immune responsiveness with age has been demonstrated most prominantly in T-cell-mediated immunity (reviews by Kay, 1980; Kay and Makinodan, 1981; Nagel, 1983; and Ackerman et al., 1991). Age-related alterations in lymphocyte function include: a decrease in T-helper, cytotoxic T cell and NK-cell activity; a decrease in T cell response to mitogens and antigens and a decrease in the production of lymphokines including IL-2 (reviewed by Ackerman et al., 1991). Age-related changes in macrophages and B cell function (Heidrick and Makinodan, 1972; Gardner and Remington, 1978; Becker et al., 1981; Goidl et al., 1983; reviewed by Ackerman et al., 1991) may also indirectly influence T-cell-mediated responses.

On the basis of the suppressor T cell theory, the normal immune system would be involved in the maintenance of self-tolerance by suppressor T cells which are stimulated by tissue-specific antigens and inhibit the activation of tissue-specific autoreactive T cells. A dysfunction of organ-specific suppressor T cells in the immune system would be a risk factor for the development of organ-specific autoimmune diseases. For instance, thymic dysfunction may cause the elimination of suppressor T cells from the immune system (Tauchi and Nishizuka, 1987; Taguchi et al., 1986; Taguchi et al., 1990) and result in autoimmunity. One of the anatomically most prominant age-related changes is the decline in size of the thymus (Boyd, 1932). There is evidence to suggest that the loss of the ability to discriminate self from nonself is also impaired with age (Zinkernagel, 1978) and could contribute to the increase in autoimmunity with age.

With increasing age beyond 65, progressively greater percentages of otherwise healthy individuals show abnormal neurological signs. Both in humans and animals the brain shrinks with advancing age with decrements occuring in brain weight, cortical thickness and white matter volume, the major loss in volume being attributed to shrinkage of neurons (reviewed by Berg and Morris, 1990). It is hypothesised that with ageing the immune system loses its ability to recognise elements of the host's body and that antibrain antibodies may be causes of neuronal degeneration (Reisberg, 1983). One of the changes associated with increasing age noted in the human CNS is a decrease in neuronal numbers. This neuronal loss results in astrocytic gliosis and spongiosis (loosening of the basic structure of the cortex due to the appearance of vacuoles of the cortex) (Rosenberg, 1983). Aged individuals demonstrate an increase in the incidence of serum autoantibodies, although this increase does not necessarily correlate with the presence of autoimmune disease (reviewed by Nagel, 1983). Brain-reactive antibodies could interact with myelin components (McFarland, 1988; Waksman, 1988) or glial elements (Allerand and Yahr, 1964). This interaction could result in neuronal death, demyelination, and membrane changes resulting in changes in permeability of the BBB (Jankovic, 1985; Jankovic et al., 1987; Forster and Lal, 1991).

1.3.5.1 Neuroendocrine-immune network in ageing

With advancing age, there is a reduction in the concentration of membrane receptors for glucocorticoids (Roth, 1975) and catecholamines (Schocken and Roth, 1977). The altered functional responsiveness of old cells to glucocorticoid action may actually be due to this defect in cell receptors (Roth, 1979). Alterations in the neuroendocrine system may also cause functional modifications of immune reactivity (Fabris, 1981) as lymphoid cells have receptor sites for hormones (Gavin, 1977; Melmon et al., 1977).

Noradrenergic sympathetic innervation is found in all primary and secondary lymphoid organs of the immune system (Felten et al., 1987; reviewed by Felten and Felten, 1991 and Ackerman et al., 1991). Noradrenergic sympathetic nerves, through the direct innervation of cells in lymphoid tissue, may provide a link between the central nervous and immune systems throughout ontogeny, maturation and senescence. Coutinho et al. (1991) demonstrated that MHC class II expression on bovine brain endothelial cells was inducible by IFN-γ and further stimulated by catecholamines through activation of β-adrenergic receptors. Because the brain endothelial cells are strategically located at the interface between the circulation and the CNS, they could be involved in the initiation of the immune response. A role for noradrenergic sympathetic innervation has also been suggested in immune senescence due to the similarity between the alterations in immune responses with age and the effect of acute sympathetic denervation in young

adults (Ackerman et al., 1991). For example, ageing and noradrenergic sympathetic denervation both result in diminished T cell responses and primary responses to T-dependent antigens and increased immunoglobulin secretion in response to polyclonal B cell stimulation. They concluded that the decreased availability of norepinephrine in secondary lymphoid organs may result in immune senescence with ageing (Ackerman et al., 1991). Possibly the noradrenergic central neurones directly control the immunologic activity of the BBB endothelium. It was suggested by Fabris (1982) that the reduced catecholamine receptor density may represent an adaptive phenomena toward the increased plasma levels of noradrenaline which increases with age (Ziegler et al., 1976). The thymus may also exert a widespread influence on the neuroendocrine system.

Sex steroid hormones influence normal immune mechanisms (Cohn, 1979) and development of autoimmune disease (Talal et al., 1984). There is a marked predominance of autoimmune diseases in females and in autoimmune models, androgens have been found to suppress and estrogens to accelerate disease severity (Roubinian et al., 1978; Ahmed and Penhale, 1982). In murine lupus, a model for systemic lupus erythematosus (SLE), females have a more severe form of the disease (Roubinian et al., 1977). Castrated males develop an accelerated autoimmune disease indistinguishable from females (Roubinian et al., 1978). However, castration of females fails to improve disease severity (Raveche et al., 1979) suggesting that it is androgen which is causing the immunosuppressive effect in this model of autoimmunity.

There are four naturally occuring animal models for neurological disease which all have a viral etiology: canine distemper encephalomyelitis (Wieniewski et al., 1972; Raine, 1976b), visna (Georgsson et al., 1982), mouse hepatitis (JHM) virus encephalomyelitis (Lampert et al., 1973; Fleury et al., 1980) and Thieler's virus encephalomyelitis (Dal Canto and Lipton, 1975). It is evident so far that demyelination can occur as a result of a viral infection and possibly the age-related or disease-associated breakdown of the BBB further results in subsequent inflammation with demyelination. In the next section, I will give an overview of MS and discuss different possible causes.

1.4 Multiple Sclerosis, an inflammatory, demyelinating disease

1.4.1 *Historical Note*

Jean-Martin Charcot (1825-1893) is given credit for establishing MS as a pathologic and clinical entity, referring to the disease as "la sclerose en plaques disseminees" in his 1868 lectures at la Salpetriere in Paris. Charcot viewed the disease as predominantly of the white matter of the CNS, with lesions of varying age and size distributed throughout the neuroaxis from the cerebrum to the spinal cord. Charcot

recognised complete and partial remissions as well as a chronic progressive phase with clinical features referring to the different lesions occurring at different times in the course of the illness (Kurtzke, 1988).

1.4.2 *Clinical Aspects of Multiple Sclerosis*

There are no laboratory tests that are diagnostic of MS and the clinical diagnosis is dependent on history and neurological examination (Traugott and Raine, 1984). The safest diagnostic criteria is considered to be that of the Schumacher Panel prepared in 1965 (Schumacher et al., 1965). Certain tests of the CSF and electrophysiological tests may strengthen the clinical diagnosis. For instance, protein content, type of lymphocytes present and the amount of IgG are important determinants in the CSF in MS. Electrophysiological tests of visual, brainstem and sensory pathways (Chiappa, 1980; Kjaer, 1980), computerised tomographic (CT) scanning (Sears et al., 1978; Mastaglia and Cala, 1980) and MRI may also demonstrate multiple lesions indicative of MS.

MS affects young adults with the majority of cases occuring between the ages of 20 and 40 (Kurtzke, 1970; McAlpine et al., 1972) and showing a female to male ratio of 1:1.5 (McAlpine et al., 1972). MS usually occurs as a series of attacks separated by periods of partial or complete remission and frequently followed by a phase of chronic progression. Alternatively the disease may progress inexorably from the start (McAlpine et al., 1972). Many MS patients have a relapsing/remitting course during the first year of the disease and then switch to a progressive one (Arnason et al., 1988).

MS primarily manifests as impaired sensory or motor performance due to the demyelination of motor axons which interferes with impulse conduction and therefore with perception and proper motor coordination (Traugott and Raine, 1984). Although each skeletal muscle fiber from mature mammals are innervated by only one motor neuron, each motor neurone innervates more than one muscle fiber. Consequently, the term "motor unit" refers to a motor neurone in the spinal cord and the muscle fibers that it innervates. Most diseases of the motor unit cause weakness and wasting of skeletal muscles (Rowland, 1985). The clinical features of MS refer to the different lesions occuring at different times in the course of the illness. Disturbances in balance are a composite complaint due to cerebellar or sensory changes as well as brainstem, auditory or visual involvement; sensory loss, weakness, micturition impairment and motor symptoms such as heaviness or dragging of the legs, undue fatigue, tripping, parapesis, quadripesis all denote spinal cord damage (Traugott and Raine, 1984).

1.4.3 *Pathology of Multiple Sclerosis*

In MS, there is an initial accumulation of leukocytes (inflammation) and fluid (oedema) around the blood vessels that lie within the CNS. Demyelination subsequently occurs in these areas of inflammation.

1.4.3.1 Inflammation

MS is an inflammatory disease where there is a localized immune response in the CNS with perivascular lymphocytic infiltrates and macrophages associated with focal zones of demyelination. MHC class II positive activated macrophages and T cells are the predominant immune cells (Traugott et al., 1983). There are immunoregulatory defects in T cell function associated with T and B cell hyperactivity with these defects accompanied by migration of activated T cells from the peripheral blood to the CNS (Hafler and Weiner, 1989). Total lymphoid irradiation of the peripheral immune compartment ameliorates progressive MS supporting the argument for the CNS inflammatory response being closely linked to the peripheral immune compartment (Hafler and Weiner, 1989).

1.4.3.2 Demyelination

The resulting myelin breakdown with the preservation of axis cylinders is the hallmark of MS (Waksman, 1984). The impaired sensory or motor performance experienced by MS patients is due to the demyelination of motor axons. Demyelination ultimately interferes with impulse conduction and therefore with perception and proper motor co-ordination. Some remyelination occurs, though if damage is severe, astrocytes proliferate (gliosis) and lay down glial fibers, ultimately forming a dense scar in the area of demyelination (Raine, 1978a; Soffer and Raine, 1980). Hence, the name multiple sclerosis, which literally means many damaged areas filled with sclerotic (scar) tissue. Glial fibrillary acidic protein (GFAP), a component of glial filaments and a useful index of astrocytic reactions and gliosis in pathological tissue (Raine, 1985) is present in MS plaques (Eng et al., 1971; Eng, 1980).

Oligodendrocytes disappear in the MS plaque (Lumsden, 1951) and it is possible that the target of the disease process is the oligodendrocytes which produce and maintain myelin, rather than the myelin itself. The relatively tenuous connection with the oligodendrocyte cell body, together with the fact that numerous myelin sheaths are produced by one glial cell in the CNS, have been invoked as possible explanations for the relative paucity of remyelination following damage to CNS myelin. The possible attempt by oligodendrocytes to remyelinate is evidenced by the "shadow plaques" which are found at the edge of MS lesions. These are composed of thinly remyelinated axons (Powell and Lampert, 1983). It was once presumed that mature oligodendrocytes in the CNS were postmitotic and therefore unable to proliferate in response to trauma. Ludwin

(1984) has shown by the *in vivo* incorporation of tritiated thymidine (a marker of cell division) that mature oligodendrocytes do actually proliferate in response to trauma. Although oligodendrocytes are able to divide in response to injury (Aranella and Herndon, 1983) their ability to achieve remyelination would be dependent on many factors.

1.4.3.3 Role of T cells

A small number of lymphocytes are usually found throughout the normal appearing white matter in MS (Traugott, 1983; Woodroofe, 1986; Hayes, 1987). Studies using monoclonal antibodies directed against T cell subsets and class II MHC-positive cells indicate that the predominant immune cells at the plaque site in MS are activated macrophages and CD4+ T cells (Pettinelli and McFarlin, 1981; Bernard and McKay, 1983; Holda and Swanborg, 1982; Traugott et al., 1983; Ben-Nun et al., 1981; Hauser et al., 1984b; Sobel et al., 1988). The number of cells increases during the acute phase with T cells predominating with phenotypic analysis showing increased ratios of $CD4^+$ to $CD8^+$ T cells (Hauser et al., 1983). There is considerable fluctuation of peripheral blood T cell subset concentrations seen in longtitudinal studies of MS patients and their close relatives (Massman Rose et al., 1985; Hughes, 1986). Whatever the etiology of MS, T cells and macrophages contribute significantly to the pathophysiology of the disease.

1.4.3.4 Immunoglobulin Concentrations

There is an increase in the concentrations of all the immunoglobulin isotypes, and in particular IgG in the CSF (Waksman, 1984; McFarlin et al., 1987) in at least 80 percent of patients with MS (Kabat et al., 1950; Tourtellotte, 1971). These immunoglobulins are generally oligoclonal (Ebers, 1984) and the continual synthesis of oligoclonal bands in the CSF of MS patients may be explained by the presence of plasma cells in MS plaques. As the disease progresses, plasma cells are found in increased numbers in normal appearing white matter (Prineas, 1985). As the antigens related to the restricted bands of antibody have not been identified it may be that the IgG is the product of a non-specific response by non-specific B cells attracted into the CNS and is a reflection of antibody production against antigen(s) residing within the CNS.

In many neurodegenerative disorders, a higher concentration of MBP, or its fragmented peptide are also found in the CSF compared to the blood (Cohen et al., 1980; Gupta, 1987). In MS, levels of MBP and its antibody in the CSF correlate with the course of demyelination, suggesting a localised sensitisation of lymphocytes to MBP within the CNS compartment (Patterson et al., 1981). MBP-reactive T cell clones and lines have been raised from the CSF of MS patients (Richert et al., 1983; Tournier-

Lasserve et al., 1986). However, whether or not the MBP-specific T cells are a secondary phenomenon that result from myelin breakdown products being released, remains unclear as MBP or MBP fragments have also been found in the sera of clinically well humans (Paterson et al., 1981).

1.4.3.5 The Role of Cytokines

The increased number of leukocytes in demyelinating regions provides the CNS with a potential source of cytokines. The production of cytokines is significantly increased during an immune response (Arai et al., 1990) and these polypeptides may orchestrate the complex processes of inflammation and immune reaction.

Abnormalities in the production of IL-2, *in vitro* inducibility of IL-2 secretion, expression of IL-2 receptors, or IL-2 responsiveness have been reported in a variety of disorders and age-associated immune dysfunctions (reviewed by Kroemer et al., 1990). IL-2 induces the production of IFN-γ, TNF-α and IL-6 *in vitro* and *in vivo* (Heslop et al., 1989; Kasid et al., 1989; Jablons et al., 1989) all of which are implicated in the pathogenesis of inflammation. In MS, elevated levels of circulating IL-2 have been found during relapse (Adachi et al., 1989) and in the CSF (Trotter et al., 1988). The immunosuppressive effect of cyclosporin A (CsA) is possibly related to its capacity to inhibit IL-2 and IFN-γ gene expression *in vitro* and *in vivo* (Kronke et al., 1984; Granelli-Piperno, 1990; Kroemer et al., 1990). Although IL-2 may have a predominantly immunostimulatory function, it may also be involved in inhibitory effects related to its actions on different populations of (suppressor, helper and effector) immune cells.

There is an increase in the number of episodic MS attacks suffered by patients being treated intravenously with IFN-γ (Panitch et al., 1987). Previous research has shown increased levels of TNF-α in the blood and spinal fluid of MS patients (Merrill et al., 1989) with TNF-α+ cells present in MS plaques (Hofman et al., 1989). The majority of these TNF-α+ cells are morphologically identified as reactive fibrous astrocytes. TNF-α is suggested to exert cytotoxic effects on oligodendrocytes resulting in demyelination (Robbins et al., 1987; Lieberman et al., 1989). It is possible that TNF-α secreted by astrocytes and macrophages may activate an array of immune defences and cause the initiation of inflammation and disease progression.

1.4.3.6 The role of astrocytes

MS is a disease in which intense fibrous astrogliosis is a prominant feature (Raine, 1983, 1984) and it is possible that astrocytes contribute to tissue damage by acting as either auxillary or effector cells. Active and replicating astrocytes are prominant in the MS lesion (Adams, 1983) where they fill areas formerly occupied by myelinated axons (Raine, 1985). The presence of myelin fragments or myelin degradation products

in astrocytes suggests that they can also act as scavenger cells (Raine, 1982). Class II MHC antigens are expressed in the brains of patients with MS (Traugott et al., 1985). However, the nature of the antigen-presenting cell has been suggested by some researchers to be macrophages (Hauser et al., 1986; Boyle and McGeer, 1990) while others suggest astrocytes (Traugott and Raine, 1985; Woodroofe et al., 1986). Traugott (1988) hypothesised that early in MS lesion formation, lymphokines released by infiltrating activated T cells lead to astrocytic proliferation and hypertrophy. In addition, locally produced γ-IFN induces Class II MHC antigen expression on astrocytes and thus contributes to local antigen presentation. Subsequently, hypertrophic astrocytes being rich in hydrolytic enzymes can participate in demyelination. In support of this hypothesis, one of the most striking features of the MS plaque is the presence of fibrillary gliosis in the areas of myelin loss (Raine, 1985).

1.4.4 *Epidemiology of Multiple Sclerosis*

The cause of MS is still unknown, however, epidemiologic studies strongly support the hypothesis that the MS process is triggered in genetically predisposed individuals by exogenous factors. Genetically, MS is strongly associated with products of genes that code for the human leukocyte antigen (HLA) of the MHC (Bertrams et al., 1972; Maito et al., 1972; Jersild et al., 1975 ; reviewed in Compston et al., 1986). The higher concordance rate for MS in monozygotic compared with dizygotic twins provides strong evidence for a genetically determined susceptibility (Mackay and Myrianthopoulos, 1966; Kuwert, 1977). In support of the hypothesis for exogenous factors, it has been found that MS prevalence varies with latitude (Kurtzke, 1977; Hallpike , 1983) and migrants between low-and-high prevalence areas carry their prevalence with them if they migrate after puberty (Kurtzke et al., 1970; Dean and Kurtzke, 1971; McAlpine et al., 1972; Alter et al., 1978). Also, there was an epidemic of MS in the Faeroe Islands after the arrival of British troops in 1940 suggesting a contagious factor (Kurtzke et al., 1986; Currier et al., 1982).

1.4.5 *Possible Role of Viral Infections in Multiple Sclerosis*

It has been suggested that MS may be caused by either a "slow virus" or one or more of the common viral infections of childhood. Waksman (1988) suggests that infection with common viruses probably initiates both the primary MS process and most exacerbations. Viral infections followed by demyelinative disease have been thought to induce autoimmunisation to myelin antigens. In human subjects with postinfectious encephalomyelitis after measles, rubella and varicella, the disease process correlates with lymphocytic sensitisation to MBP (Johnson et al., 1981). These results may testify to past infection and a possible role in autoimmunisation. Alternatively, a persistent

infection of the nervous system with an associated immune response to the pathogen may be the cause. Measles, herpes simplex, cytomegalovirus, rubella, coronavirus and lymphocytic choriomeningitis have been isolated from the brains of MS patients (Bauer et al., 1980; Boese, 1980) but no common etiology has emerged from these studies. Despite the fact that neither a virus nor antigen have been found many still support the viral model (Wege et al., 1984; Tardieu et al., 1984).

1.4.5.1 Similarities between poliomyelitis and Multiple Sclerosis

The pattern of epidemiologic findings in MS is reminiscent of the pattern of poliomyelitis in the early part of the 19th Century (Poskanzer et al., 1980; Nathanson et al., 1979, reviewed by Waksman, 1988). When polio infection occurs in early childhood, gastrointestinal disease develops, usually without CNS involvement. However, polio infection occuring in adolescence or early adult life frequently results in neurological dysfunction as well. The probability of viral transmission in early childhood diminished as domestic hygiene improved in developed countries and with the decrease in the number of children in each family. This resulted in an increase of poliovirus infections occurring with neurologic involvement, particularly in countries in the temperate zone (latitude effect) and in higher socioeconomic groups within the affected populations. These features are also seen in MS. The latitude effect is evident (Kurtzke, 1977; Hallpike , 1983) with an increased incidence among higher socioeconomic groups. In the Faroe Islands epidemic, possibly an infectious agent was carried in by British troops. There has been a link made between severe viral infections of Faroe Island adolescents between the ages of 13 to 26 in this period and susceptibility to MS (Currier et al., 1982; Kurtzke et al., 1986)

MS patients have been found to experience infection with common exanthematic and other viruses significantly later in life, eg. during late childhood or early adolescence than matched controls (Sullivan et al., 1984; Anderson et al., 1985; Compston et al., 1986). Since children in the tropics are regularly infected with measles, mumps and rubella before one year of age, it seems likely that both the low incidence of MS in the tropics and the resistence to MS of migrants from the tropics may be directly related to the early age of infection with such world-wide agents (Alvord, 1988). Thus, this data suggests that a combination of genetic predisposition and environmental factors (possibly a viral infection) experienced in adolescent life leads to the development of MS.

1.4.6 *Abnormalities in the vascular system*

Lesions of MS are associated with small blood vessels (Adams, 1972) and it was at one time proposed platelet changes *in vivo* may cause venular occlusion and contribute to plaque formation (Putnam, 1935, 1937; Fog et al., 1955) with early lesions of MS due

to clots in small veins or a result of paralysis or spasm of small vessels. Alterations of the clotting mechanism have been reported in MS patients in *in vitro* studies (Nathanson and Savitsky, 1952; Caspary et al., 1965; Wright et al., 1965; Millar et al., 1966) and experimental work in animals producing blockage of small veins supported this idea. However, the notion of venular thrombosis as the pathogenic factor in MS plaque formation has been discarded (Putnam, 1935, 1937; Fog et al, 1955). Anti-coagulants such as dicoumarol, heparin and atromid, vasodilators such as histamine, tetraethylammonium chloride and hydergine and circulatory stimulants such as ephedrine, caffeine, alcohol, adrenal cortical extract and deoxycorticosteroneacetate have been tested and appear to be ineffective in the treatment of MS (Aronson et al., 1982).

1.4.7 *Age related factors in MS*

MS is an age dependent disease. It is rare for children or adults over the age of 50 to develop MS (Kurland, 1952). Ageing is associated with marked deterioration of the immune system (Gardner, 1980; reviewed by Talor and Rose, 1991) and a significant increase in the incidence of autoimmune diseases (Talor and Rose, 1991). Mechanisms to explain the increase in autoimmunity have been: a repeated or prolonged insult by endogenous autoantigen; molecular mimicry with environmental antigens; or a decrease in immune suppression with age (Talor and Rose, 1991).

Dore-Duff et al. (1987) found age-related changes in lymphocyte adherence to myelinated cerebellar tissue in MS patients with adherence levels to myelin being significantly higher than in controls at most ages tested. Maximum adherence to myelin was found in both MS and control cells during the ages of 20 to 45 years which corresponds to the peak age of onset of MS. These findings could be explained by a number of possibilities: age-related differences in human myelin, possibly the receptor for T cells on myelin is altered with ageing; there could be an increase in the number of cells that recognise myelin; or a decline in immunoregulatory mechanisms governing adherence of lymphocytes. There is subsequently a decrease in the adherence levels of lymphocytes to myelin from elderly donors. This also correlates with the decrease in the incidence of MS in older patients (Kurland, 1952) and the abating of disease activity in some older patients.

1.4.8 *MS Drug Therapy*

Many attempts have been made to modify the course of MS. Some drug therapies have been directed at controlling the associated side effects of this disease whereas others have been palliative in nature and design (Mertin, 1985). With an increasing understanding of cellular immunology, more recent treatments have concentrated on immunomodulation and/or anti-inflammatory actions (Mertin, 1985).

Adrenocorticotropic hormone (ACTH), prednisone and its derivatives azathioprine and cyclophosphamide are the most widely used immunosuppressive drugs (Ellison and Myers, 1980). Unfortunately, no treatment has been shown to alter the progressive tissue damage of MS in the nervous system although it is possible to moderate the severity of attacks. Short-term high-dose therapy with ACTH or prednisone can shorten the duration and diminish the severity of acute attacks, however long-term moderate dose therapy is ineffective in modifying the disease (Traugott and Raine, 1984). ACTH is extracted from beef pituitary glands whereas adrenal corticosteroid hormones are extracted from beef adrenal glands. These act pharmacologically like the corresponding human hormones. Physiologically, ACTH acts by stimulating the adrenal gland to release "glucocorticoid" hormones. They reduce oedema and other aspects of inflammation, cause destruction of some types of lymphocytes and improve conduction of nerve impulses in demyelinated nerve fibres. Both drugs have adverse side effects and cannot be used chronically. The complications of ACTH and glucocorticoid therapy are generalised puffiness, psychosis, ulcer, general infections, acne, abnormalities of sodium and potassium levels, softening of bone and pathologic fractures, cataracts, hypertension, diabetes and adrenal exhaustion (Wilkinson, 1988).

Although corticosteroids are effective in treating acute exacerbations, they do not influence the progression of disease, whereas high dose short-term cyclophosphamide may be of some benefit in slowing progression. MS can be arrested in the majority of patients tested for a year or more by this powerful immunosuppressive agent (Hauser et al., 1983). Cyclophosphamide interferes with nucleic acid metabolism and at high doses kills lymphocytes. Consequently, a combination of ACTH or corticosteroid (used both for anti-inflammatory and immunosuppressive actions) and a longer term immunosuppressive (azathioprine, cyclophosphamide) is often used to treat the disease (Dau et al., 1980).

Currently, anti-inflammatory drugs or general immune system suppressants such as steroids or cyclosporin A are commonly used in MS. These drugs are expensive and have severe side effects. For these reasons, physicians are reluctant to prescribe them in the early stages of autoimmune disease when they might do the most good. Experimental models such as EAE have been used to study neuroimmunological relationships in the CNS and to test anti-inflammatory and immunosuppressive agents which may be useful in the treatment of MS. In the next section I will characterise EAE as an experimental model considering the relative role inflammatory cells play in disease manifestation.

1.5 Experimental Autoimmune Encephalomyelitis - an experimental model of inflammation of the CNS

Experimental Autoimmune Encephalomyelitis (EAE) is an inflammatory disease of the CNS produced in laboratory animals by the injection of neural tissue in adjuvant (Hashim et al., 1980; Raine, 1984). It is an autoimmune condition in which the host animal is induced to mount an immune attack on constituents of myelin (Paterson, 1976, 1981; Paterson et al., 1981). In some respects, EAE immunologically, pathologically and clinically resembles the human demyelinating disease MS (reviewed by Raine, 1985) and it has since become the principal animal model for the disorder.

1.5.1 *Historical Note*

With the introduction of the anti-rabies vaccine by Pasteur in 1885, there appeared reports of "neuroparalytic accidents" in some of the patients vaccinated. These patients exhibited weakness and sensory disturbance in the limbs, sphincter dysfunction and perivascular inflammation and demyelination in the CNS (Bassoe and Grinker, 1930). The Pasteur vaccine consisted of repeated subcutaneous injections of desiccated spinal cords removed from rabbits which had been infected with living fixed rabies virus (Harvey and Acton, 1923). Many investigators studied the effect of injections of nervous tissue into experimental animals (reviewed by Hurst, 1932) and it was suggested that the "neuroparalytic accidents" following the rabies vaccination were due to neural contamination of the vaccine (Koritschoner and Schweinburg, 1925).

In 1933, Rivers et al. induced the disease in monkeys by the repeated intramuscular injection of brain extracts. Diseased animals clinically exhibited ataxia and weakness and were found to have perivascular inflammatory and demyelinated lesions in the CNS. The induction of disease was facilitated with the introduction of adjuvants into the inoculum. Induction of EAE without adjuvant requires multiple injections of CNS material. For instance, Rivers et al. (1933) injected normal rabbit brain 94 times into 8 monkeys to achieve clinical and histological evidence of EAE in only 2 monkeys. Using complete Freund's adjuvant (CFA) supplemented with mycobacterium (Freund and McDermott, 1942), EAE symptoms were evident in monkeys within 2 weeks (Morgan, 1947; Kabat et al., 1947) and EAE became a practical laboratory disease model to study demyelinating diseases. Subsequently, the disease has been induced in a variety of animals (reviewed by Waksman, 1959) with several different forms of EAE being distinguished by pathological findings and clinical profiles.

1.5.2 *Clinical aspects of EAE*

The chief clinical manifestation of EAE is an ascending paralysis. In susceptible animals, EAE is generally characterised by transient ataxia, hindlimb weakness or paralysis and urinary and fecal incontinence due to autonomic nervous system dysfunction from spinal cord injury (Paterson, 1959, 1966, 1971). Specifically, in the monkey, clinical features consist of ataxia, limb weakness and paralysis, tremour, spasticity, visual loss, ptosis, facial weakness and nystagmus (Rivers et al., 1933; Rivers and Schwentker, 1935; Ferraro and Jervis, 1940; Morgan, 1947; Kabat et al., 1947) whereas weight loss, transient ataxia, limb weakness and paralysis, and incontinence have been described as the main clinical features of rabbits (Morrison, 1947; Waksman and Adams, 1955; Waksman and Adams, 1956), rats, mice and guinea-pigs (Freund et al., 1947; Lipton and Freund, 1953; Waksman and Adams, 1956)

1.5.3 *Genetic variations in EAE*

An association between MHC compatibility and susceptibility to EAE has been reported in guinea pigs (Kies et al., 1975; Lisak et al., 1975; Webb et al., 1973) and rats (Gasser et al., 1973, 1975; Williams and Moore, 1973; Gunther et al., 1978; Wettstein et al., 1981). Survival rates of monkeys treated with antibodies to class II MHC molecules is significantly higher than controls after induction of EAE (Jonker et al., 1988). Administration of anti-class II MHC antibodies at the onset of clinical signs of EAE in mice prevents the homing of radio-labelled neurospecific T cells to the brain (Sriram and Carroll, 1991). Konno et al. (1990) demonstrated that if class II MHC positive microglia are induced in recipient rats by corticol cryoinjury or eyeball enucleation prior to induction of EAE, the lesions which are similar to lesions found normally in EAE develop in accord with the class II MHC-positive microglia. This suggests that class II MHC-positive cell clusters can serve as a target for autoimmune CNS lesions. This class II MHC association is also noted in many autoimmune diseases and indicates that CD4 T cells recognising antigen in association with class II MHC products are involved in the disease pathogenesis.

1.5.4 *Pathology of EAE*

Generally, the disease is characterized by leukocyte infiltration of the brain and spinal cord, oedema (Raine, 1984; Kerbro de Rosbo et al., 1985; Vanderbark et al., 1985; Sedgewick et al., 1986; Simmons et al., 1987) and astrocytic hypertrophy (Bubis and Luse, 1964; Lampert, 1965; Hirano et al., 1970; Raine et al., 1980; Blakemore, 1979). There is some demyelination of the spinal roots (Pender, 1987), with little demyelination of central axons (Kerbro de Rosbo et al., 1985; Vanderbark et al., 1985; Pender, 1987). Although, typically perivascular inflammatory lesions are disseminated

throughout the CNS affecting the white matter more than the grey matter (Paterson, 1966; Alvord, 1970; Palmer and Dawson, 1969), this distribution varies in different species. In the rat, mouse and guinea-pig, lesions are evident in both the brain and the spinal cord. The spinal cord of the rabbit is more severely damaged than the brain, whereas, in the monkey, lesions are predominantly found in the brain, brainstem and cerebellum (reviewed by Waksman, 1959).

EAE can be actively induced in genetically susceptible species by immunisation with neural tissue derived antigens emulsified in adjuvant (Paterson, 1981; Paterson et al., 1981; Hickey et al., 1983) or by the transfer of lymphoid cells from actively immunised donors into naive syngeneic recipients (Paterson, 1960; Richert et al., 1979; Ben-Nun et al., 1981; Hickey et al., 1987; Holda et al., 1980; Panitch, 1980) (Fig. 1.5).

1.5.5 *Active induction of EAE*

EAE is actively induced in susceptible animals by the inoculation of encephalitogen such as MBP in CFA. The usual inoculum consists of brain and spinal cord white matter, whole spinal cord or purified MBP (50µg/animal) homogenised in saline and emulsified 1:1 in CFA to which Mycobacterium butyricum has been added (400µg/animal). Induction of EAE can be by subcutaneous or intracutaneous inoculation (Raine, 1984) and is usually in the footpads (Raine, 1985).

MBP is an 18,000 molecular weight molecule made up of 170 residues (Carnegie and Lumsden, 1966, 1967) and different segments of the molecule are encephalitogenic in different species (Raine, 1976). The encephalolitogenic sequences are known for the rat (Dunkley and Carnegie, 1974) with two peptides being active, one small (sequence 44-48) and one large (sequence 71-85) (Martenson et al., 1972). Lewis rats only respond to sequence 75-84 (Hashim et al., 1978). Guinea pig MBP (GPMBP) is the most encephalitogenic for Lewis rats, being 25 times more active than bovine, human or rabbit MBP and 10 times more active than rat MBP (McFarlin et al., 1973).

CFA is thought to result in better antigen presentation because it acts as a "depot" to slowly administer the antigen (Raine, 1985), which would otherwise disperse quickly and lose its ability to stimulate and to boost both delayed type hypersensitivity and antibody responses. In addition, bacterial products would further activate an effective immune response. The addition of Bordetella pertusis in adjuvants results in increases in the number of lymphocytes as well as activating macrophages which results in a marked increase in the antibody response (World Health Organisation, 1976). EAE can be induced in rats with MBP without the use of adjuvant or pertussis vaccine if rats are pretreated with a peritoneal irritant and by subsequently injecting MBP into pre-enlarged popliteal lymph nodes (Levine et al., 1990).

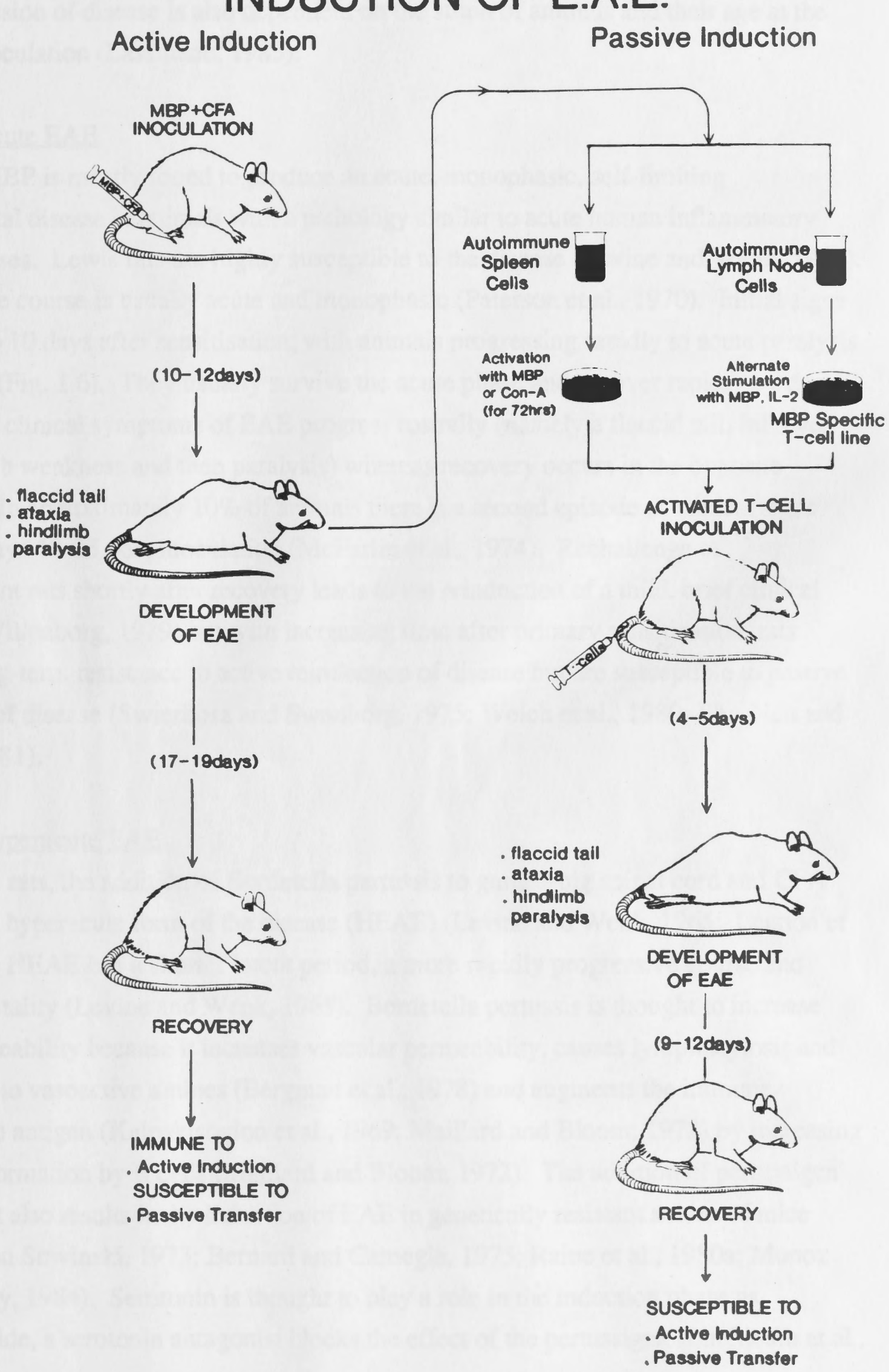


Fig. 1.5: EAE can be actively induced in susceptible species by immunisation with MBP emulsified in adjuvant or by the transfer of lymph node derived cell lines, or spleen cells from actively immunised donors into naive syngeneic recipients.

Encephalitogenicity varies with the species of neural antigen used, the amount of neural antigen and mycobacterium used, and the proportions of water and oil in the adjuvant emulsion (Lee and Sneider, 1962; Paterson and Bell, 1962; Shaw et al., 1962). The expression of disease is also dependent on the strain of animals and their age at the time of inoculation (Lassmann, 1983).

1.5.5.1 Acute EAE

MBP is mostly found to produce an acute, monophasic, self-limiting experimental disease in animals with a pathology similar to acute human inflammatory CNS diseases. Lewis rats are highly susceptible to the disease (Levine and Wenk, 1963). The disease course is usually acute and monophasic (Paterson et al., 1970). Initial signs appear 9 to 10 days after sensitisation, with animals progressing rapidly to acute paralysis by day 12 (Fig. 1.6). They usually survive the acute phase and recover rapidly by day 17 to 19. The clinical symptoms of EAE progress rostrally (namely a flaccid tail, followed by hindlimb weakness and then paralysis) whereas recovery occurs in the opposite direction. In approximately 10% of animals there is a second episode of clinical signs between days 19-25 post inoculation (McFarlin et al., 1974). Rechallenge of convalescent rats shortly after recovery leads to the reinduction of a mild, brief clinical episode (Willenborg, 1979) but with increasing time after primary sensitization, rats exhibit long-term resistance to active reinduction of disease but are susceptible to passive induction of disease (Swierkosz and Swanborg, 1975; Welch et al., 1980; Ben-Nun and Cohen, 1981).

1.5.5.2 Hyperacute EAE

In rats, the addition of Bordetella pertussis to guinea-pig spinal cord and CFA results in a hyperacute form of the disease (HEAE) (Levine and Wenk, 1965; Lennon et al., 1976). HEAE has a shorter latent period, a more rapidly progressive course and higher mortality (Levine and Wenk, 1965). Bordetella pertussis is thought to increase BBB permeability because it increases vascular permeability, causes lymphocytosis and sensitivity to vasoactive amines (Bergman et al., 1978) and augments the immune response to antigen (Kalpaktsoglou et al., 1969; Maillard and Bloom; 1972) by increasing antibody formation by B cells (Maillard and Bloom; 1972). The addition of pertussigen in adjuvant also results in the induction of EAE in genetically resistant strains of mice (Levine and Sowinski, 1973; Bernard and Carnegie, 1975; Raine et al., 1980a; Munoz and McKay, 1984). Serotonin is thought to play a role in the induction phase as methysergide, a serotonin antagonist blocks the effect of the pertussigen (Linthicum et al., 1982).

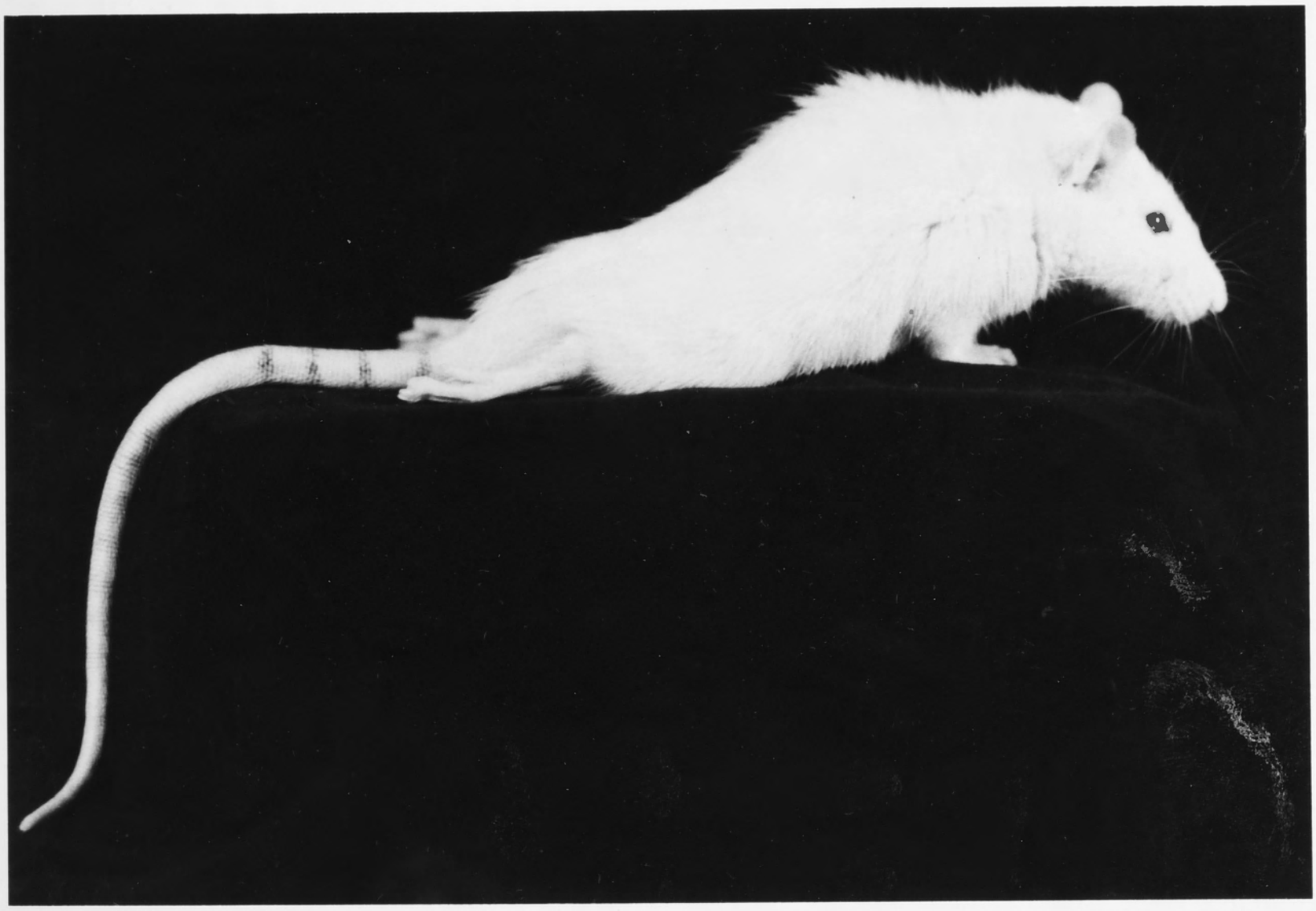


Fig. 1.6: Lewis(JC) rat exhibiting signs of EAE 16 days post inoculation with spinal cord homogenised in complete Freund's adjuvant. There are obvious signs of a flaccid tail and hindlimb paralysis (Photograph taken by N. Bowern).

1.5.5.3 Chronic relapsing forms of EAE

The neuropathological changes and disease course observed in the chronic relapsing forms of EAE (CREAE) found in Strain 13 guinea-pig (Stone and Lerner, 1965; Wisnewski and Leith, 1977), SJL/J mice (Brown and McFarlin, 1981; Lublin et al., 1981) and more recently in monkeys (Shaw et al., 1988) are more typical of MS (McKay, et al., 1973; Raine, 1976; reviewed by Lassman, 1983). The features of CREAE are inflammatory lesions, demyelination, gliosis and oligodendrocyte loss (Lassmann and Wisniewski, 1979a; Shaw et al., 1988). The demyelinated plaques are often found around small veins and usually show recent demyelinative activity with some axonal degeneration (Lassmann and Wisniewski, 1979a). CREAE is induced by whole myelin rather than MBP alone (Raine et al., 1977; Lassman et al., 1979) and overcomes the problem where EAE has an acute, monophasic and self limiting course, whereas MS has a relapsing/remitting nature.

1.5.5.4 Inflammatory infiltrate

Studies using monoclonal antibodies directed against T cell subsets and class II MHC-positive cells indicate that the predominant immune cells in EAE lesions are CD4+ (helper) and CD8+ (cytotoxic/suppressor) lymphocytes. Macrophages and Ig+ B cells are also evident in EAE lesions (Hickey et al., 1983; Traugott et al., 1985). Inflammatory cell composition also depends on the mode of immunisation (Levine, 1974) as the general pattern of cellular distribution in CNS lesions is similar for all models of EAE except where pertussis has been used (Traugott et al., 1981, 1982a, 1982b; Sriram et al., 1982; Hickey et al., 1983; Hickey and Gonatas, 1984). Animals inoculated with MBP in CFA containing Mycobacterium tuberculosis or Mycobacterium butyricum exhibit a monocytic infiltration (Traugott, 1989) whereas the use of Bordetella pertussis vaccine produces HEAE with neutrophilia (Levine and Wenk, 1964). Neutrophils are recognised as having the greatest oxidative capacity of all the phagocytes (Klebanoff, 1988).

1.5.6 *Passive induction of EAE*

EAE can be passively induced by intravenous transfer of lymphocytes from actively immunised donors to naive syngeneic recipients (Paterson, 1960; Astrom and Waksman, 1962). Passive EAE can be induced using either spleen cells from donors immunised 10 to 12 days previously with 50µg MBP in CFA and incubated with either MBP or concanavalin A (ConA) for 72 hours (method of Panitch and McFarlin, 1977) or a cell line derived from the lymph nodes of donors immunised 10 to 12 days previously with 50µg MBP in CFA. The cell line is alternately stimulated with MBP antigen and IL-2 (method of Ben-Nun et al., 1981). Activated cells are transferred into the tail vein of rats at a dose of 30 to 50×10^6 cells/recipient. Without activation by incubation with MBP

or ConA before injection into syngeneic recipients these cell populations will inefficiently transfer EAE (Richert et al., 1979). Although both ConA and MBP activated cells transfer disease, they may be effective via different mechanisms as MBP stimulation is more efficient. Only 10^6 viable cells activated by MBP are necessary for the transfer of disease, whereas 10^7 ConA activated viable cells are required (Panitch, 1980; Richert et al., 1981).

The T cell population responsible for disease transfer has been shown to be the CD4+ T cell subset in both mice and rats (Hickey et al., 1983; Traugott et al., 1985). Initial recognition of autoantigen in association with class II MHC, followed by the clonal expansion of MBP-specific $CD4^+$ cells, represents the primary events in EAE induction (Paterson and Swanborg, 1988). Development of disease after the transfer of T cells which have already been sensitised to MBP into recipient animals is a model of the effector stage of EAE (Paterson, 1960). The earlier onset of disease possibly occurs as the initial recognition and proliferative events have already taken place. The disease produced is clinically and histologically similar to that of active induction, however passively sensitised rats develop disease in 4 to 5 days. The animals subsequently recover, but remain susceptible to both active and passive reinduction of disease. The passively induced EAE model is useful in that it is free of the "antigen depot" effects which are present in the actively induced disease.

1.5.7 *Immunisation with CNS endothelial membrane fractions*

Guinea-pigs and monkeys immunised with CNS endothelial membrane fractions uncontaminated with myelin proteins can cause an EAE-like disease consisting of paralysis and demyelination (Tsukada et al., 1987). This result suggests that an immune disturbance of the BBB is sufficient to cause EAE with specificity to a parenchymal target (eg., myelin).

1.5.8 *T cell mediated disease*

EAE is a T cell mediated disease, as demonstrated by an inability of genetically susceptible animals depleted of T cell precursors by neonatal thymectomy (Wick, 1972; Bernard et al., 1976), thoracic duct drainage (Gonatas and Howard, 1974), or anti-thymocyte serum (Ortiz-Ortiz and Wiegle, 1976) to manifest symptoms. Injection of normal thymocytes restores susceptibility to disease (Arnason et al.,1962; Lennon and Byrd, 1973; Ortiz-Ortiz and Wiegle, 1976; Gonatas and Howard, 1979). The important role played by T cells in this process is further illustrated by the adoptive transfer of sensitised T cells from an EAE donor (Paterson, 1960; Stone, 1961; Astrom and Waksman, 1962; Levine et al., 1970; Bernard et al.,1976) or introduction of encephalitogenic T cell clones into naive, syngeneic recipients causing disease symptoms

(Ben-Nun et al., 1981; Hauser et al., 1984b; Raine, 1984). The course of the disease can also be modified by immunosuppressive medication (reviewed by Raine, 1985), such as treatment with anti-class II MHC antibodies or treatment with anti-CD4 antibodies. Thus it is clearly established that EAE is mediated by MHC class II antigen-restricted T cells (Pettinelli and McFarlin, 1981; Zamvil et al., 1985). However, the actual mechanisms initiating the entry of T cells and the subsequent damage remain unclear.

1.5.9 *T cell entry into the CNS in EAE*

As mentioned previously, lymphocytes are normally found in low numbers in the CNS. A critical feature in the pathogenesis of EAE is the entry of T cells into the parenchyma of the CNS. This is the case whether EAE is induced actively by immunisation with MBP emulsified in CFA or passively with activated MBP-specific CD4+ T cells (Smith and Waksman, 1969; Raine, 1976; Hickey et al., 1983; Fontana et al., 1984; Traugott et al., 1986; Zamvil and Steinman, 1990). As early as 5 days after the injection of neural antigen, T cells have entered the perivascular space in the CNS (Waksman and Adams, 1962; Traugott et al., 1978, 1981) and at the height of clinical signs, the typical EAE lesion contains approximately 60-80% T cells, 30-50% macrophages and 10-12% B cells (Sriram et al., 1982). The mechanism of this initial entry into the CNS is not known, and the concept that antigen recognition at the endothelial cell surface triggers migration would seem no longer tenable (Lassman et al., 1986; Hinrichs et al., 1987).

Hickey et al. (1983) found that the majority of the T cells in the CNS lesions are activated. Wekerle et al. (1986) have proposed that the initial emigration of lymphocytes in the CNS is not immunologically specific. They hypothesise that "activated" T cells of any specificity migrate across the CNS endothelium into the parenchyma of the CNS and that this random migration acts as an immune surveillance mechanism. If the activated cells entering the CNS encounter an antigen for which they have a specificity, they remain, otherwise they may die or move back out of the CNS.

Willenborg and Parish (1988) expanded the hypothesis that "activated" T cells of any specificity migrate across the CNS endothelium into the parenchyma of the CNS by suggesting that "activation" translated to the expression of enzymes on the lymphocyte surface. These enzymes assisted the cells in degrading the sub-endothelial basement membrane and the extracellular matrix, thus facilitating movement through interendothelial junctions and the subendothelial extracellular matrix into the parenchyma. Both direct and indirect evidence has been provided to support this hypothesis. Activated lymphocytes produce elevated levels of a heparan sulphate-specific endoglycosidase (Naparstek et al., 1984; Lider et al., 1989) which specifically degrades the heparan sulphate side chains of the proteoglycan scaffold of the extracellular matrix. Inhibitors of this enzyme also

prevent EAE development (Naparstek et al., 1984; Lider et al., 1989; Willenborg and Parish, 1988). Non-activated cells which lack the elevated levels of heparan sulphate endoglycosidase (Naparstek et al., 1984; Lider et al., 1989) do not cause disease (Panitch et al., 1981; Ben-Nun et al., 1981; Holda et al., 1980; Peters and Hinrichs, 1982).

Neurospecific cells do not need to be stimulated by their specific antigen in order to cause disease, but can also be activated in a non-antigen-specific way using the lectin ConA (Panitch, 1980; Hinrichs et al., 1981; Peters , 1982). However, activation with all T cell mitogens does not result in the ability of cells to transfer disease. Phytohaemagglutinin (PHA) is a T cell mitogen able to produce large, dividing, blast-like cells in culture (Nowell, 1960). However PHA does not enable immune cells to transfer EAE (Peters and Hinrichs, 1982; Jones et al., 1989). This may be due to the effects these lectins have on lymphocyte migration. Schlesinger and Israel (1974) tested a range of lectins for their ability to affect lymphocyte migration. Radiolabelled murine lymphocytes were exposed to ConA and PHA, and then injected intravenously into syngeneic recipients. It was found that ConA inhibited lymphocyte migration to lymph nodes to a greater degree than entry into spleen, whereas PHA inhibited migration to each of these organs to a similar degree. These results suggest that the ability of lectins to cause the transfer of disease could be related to their effects on lymphocyte migration.

The ability of the T cell to enter the CNS is not related to the antigen specificity of the cell, the phenotype of the cell, the MHC restriction element used by the cell, or even MHC compatibility with the host (Hickey et al., 1991). T cells not in blast phase were excluded from entry into the rat CNS whereas T-lymphoblasts entered the CNS in an apparently random manner (Hickey et al., 1991). Their results suggest that entrance was primarily dependent upon the activation state of the cells. It is hypothesised that in the process of activation, the T cell acquires new enzymatic and cell surface properties which enable it to enter the CNS. If they encounter an antigen in MHC context they persist to elicit an inflammatory response or otherwise exit.

In passive transfer, the fate of the activated cells following transfer to recipient animals is unknown. There is a latent period of 4 to 6 days between the injection of cells and the development of clinical signs. There may be a need for cell replication, differentiation or recruitment of recipient non-specific reactive cells. Results using gliotoxin treated lymphocytes appears to rule out a need for replication of donor cells upon transfer (Willenborg et al.,1988). Activated MBP-specific cells have been found to rapidly enter the CNS within a few hours after intravenous injection (Hickey et al., 1989; Meyermann et al., 1986; Wekerle, 1986; Wekerle and Fierz, 1985; Wekerle et al., 1986; Wekerle et al., 1987). Subsequently, it has been reported by some researchers that the numbers of activated MBP-specific cells progressively diminish until the opening of the BBB and the onset of EAE on approximately day 4 post inoculation (Hickey et al., 1989;

Meyermann et al., 1986; Wekerle et al., 1986) whereas, in contrast, Naparstek et al. (1983) reported that MBP-specific cells selectively and progressively accumulated in the CNS over the 72 hours post injection before the onset of EAE.

1.5.10 *Recruitment of non-specific lymphocytes*

Although it appears that neuroantigen-specific T cells initiate EAE, the majority of lymphocytes in established CNS lesions are not neuro-antigen-specific (McClusky et al., 1963; Dresser et al., 1970; Werdelin et al., 1971; Werdelin and McClusky, 1971; Sriram et al., 1982; Cross et al., 1990) and the recruitment of non-specific lymphocytes may be the most salient aspect of lesion formation during EAE. Ninety percent of the inflammatory cells comprising the lesions of EAE represent cells which are not specific for neuroantigen (Kosunen et al., 1963; Werdelin and McCluskey, 1971; Cross et al., 1990). MBP-specific cells are found to "home" to the CNS 24 hrs prior to and during initial clinical disease and despite massive parenchymal inflammatory cell infiltration almost invariably remain within the perivascular area (Cross et al., 1990). Hickey et al. (1991) also found peak concentrations of T cells predominantly around the meninges and blood vessels throughout the study. The fact that lesions of EAE begin around capillaries of the CNS suggests that events on the vascular endothelium are responsible for the cellular infiltrate. Sensitised lymphocytes are thought to enter the CNS, recognise specific antigen triggering the release of soluble mediators which attract and activate other inflammatory cells. Thus, MBP-specific cells are proposed to orchestrate the influx of inflammatory cells, which are mostly of recipient derivation, from the perivascular location.

1.5.10.1 Adhesion molecules in EAE

Cannella et al. (1990) examined the expression of two molecules associated with cell adhesion, a murine HEV marker (MECA-325) (Duijvestijn et al., 1986) and a murine homologue of ICAM-1 (Takei, 1985; Horley et al., 1989) on CNS vessels during chronic relapsing EAE in the mouse. Increased expression of adhesion molecules corresponded with clinical relapses and they suggested that the expression of MECA-325, ICAM-1 and MHC class II appear to be important in leukocyte homing and adhesion to the BBB during active stages of autoimmune demyelination. Subsequently, it was shown that MECA-325 is absent from normal CNS tissue but rises to maximal levels during acute disease as does ICAM-1 and MHC class II (Cannella et al., 1991). LFA-1 and CD4 receptor molecules on inflammatory cells also fluctuated in parallel with MECA-325, whereas CD8 receptor molecule was absent during the preclinical stage but was upregulated in inflammatory cells and astrocytes with acute disease activity and during relapses (Cannella et al., 1991). They suggested that cellular entry into the CNS is related

to the fluctuations of adhesion molecules. Similarly, Raine et al. (1990) in a study using monoclonal antibodies directed against different adhesion molecules on frozen sections in combination with the avidin-biotin-complex technique, found that the attachment and infiltration of MBP-specific lymph node cells and T cell lines correlated with the onset of signs and the appearance of MECA-325 and ICAM-1 on vessels and the appearance of cellular infiltrates which were also largely LFA-1+ lymphocytes. Wilcox et al. (1990) also found cells of the perivascular infiltrate in acute EAE and CREAE in guinea-pigs expressed increased levels of ICAM-1. Hence in EAE, several distinct adhesion-related molecules appear to be involved in the regulation of the cellular traffic to and from the CNS.

1.5.11 *Antigen presentation in EAE*

In EAE, antigen is presented in the context of class II MHC molecules to CD4+, CD8-, IL-2 dependent lymphocytes (Ben-Nun et al., 1981; Fritz et al., 1985; Happ et al., 1988; Hickey and Kimura, 1988; Holda and Swanborg, 1982; Sakai et al., 1985; Takenaka et al., 1986; Vandenbark et al., 1985). Class II MHC antigen appears to play a role in the pathogenesis of EAE, as treatment of antigen presenting cells with anti-class II MHC antibody blocks T-cell proliferation to MBP *in vitro* and prevents relapses in mice *in vivo* (Steinman et al., 1981; Sriram and Steinman, 1983). Increased endothelial cell class II MHC expression correlates with the development of increased permeability of the BBB (Weigler, 1980) facilitating cellular infiltration into the CNS. The antigen presenting cell is unknown and the astrocyte, oligodendrocyte, microglial and endothelial cells have all been suggested.

1.5.11.1 The role of microgial cells

Hickey (1991) suggests that the vasogenicity of the inflammatory infiltrate could be explained if activated T cells entering the CNS, produce a wide spectrum of cytokines including IFN-γ and thus induce MHC expression on perivascular microglial cells. T cells can induce MHC expression (Pober et al., 1983) and this could also explain the phenomenon where activated encephalitogenic T cells could induce EAE if injected intravenously but not if given intrathecally. The need for activated cells to cross the BBB to induce disease could be explained by the necessity for perivascular antigen presentation. Allen et al. (1987) also suggested that a macrophage/monocytic antigen presenting cell was necessary for the development of lymphocytic choriomeningitis in mice.

1.5.11.2 The role of astrocytes

Although the T cell derived lymphokine γ-IFN induces astrocytes to express class II molecules (Fierz et al., 1985; Hirsch et al., 1983; Wong et al., 1985) and astrocytes are capable of presenting MBP to appropriately sensitised T cell lines *in vitro* (Fontana et al., 1984, 1987; Wekerle, 1986; Wekerle et al., 1987), researchers using *in vivo* methods have found that astrocytes expressing class II MHC are either very rare or unable to be detected in animals developing EAE (Sobel et al., 1984b; Sobel and Colvin, 1985; Craggs and Webster, 1985; Hickey et al., 1985; Lassmann et al., 1986; Matsumoto and Fujiwara, 1986; Matsumoto et al., 1986; Vass et al., 1986). These results suggest it is unlikely that astrocytes play an important role in MBP antigen presentation in the CNS.

1.5.11.3 The role of oligodendrocytes

Oligodendrocytes have also been found to express substantial amounts of class II MHC molecules *in vivo* (Suzimura et al., 1986), *in situ* (Ting et al., 1981) and during virus infection (Rodriguez et al., 1987). Despite this, immunohistochemical studies have not detected class II MHC molecules on oligodendrocytes in developing EAE, in the normal human CNS, or in patients suffering from MS (Craggs and Webster, 1985; Hayes et al., 1987; Matsumoto and Fujiwara, 1986; Matsumoto et al., 1986; Traugott et al., 1985; Vass et al., 1986), again suggesting that these cells do not play a significant role in antigen presentation in EAE.

1.5.11.4 The role of endothelial cells

Despite its interaction with lymphocytes during their entry into the CNS, there is no evidence to suggest that the endothelial cell is the antigen presenting cell in the development of CNS inflammation and studies using rat bone marrow chimeras have demonstrated that the endothelial cell cannot be the antigen presenting cell in rodents (Hickey and Kimura, 1988; Hinrichs et al., 1987; Hickey, 1990). Although some reports have been unable to find class II MHC positive endothelial cells in EAE and MS (Hayes et al., 1987; Matsumoto and Fujiwara, 1986; Matsumoto et al., 1986; Lassmann et al., 1986; Vass et al., 1986) there have been some reports where CNS endothelium has become class II MHC positive in EAE and MS (Hickey and Kimura, 1987; Hickey et al., 1985; Lampson and Hickey, 1986; Sobel et al., 1984a; Sobel and Colvin, 1985; Traugott et al., 1985; Hickey , 1990). In addition, in vitro studies have shown that endothelial cells can present antigen to MBP-specific lymphocytes in a class II MHC restricted way (Male and Pryce, 1988,1989; Male et al., 1987; McCarthy et al., 1985; Pober et al., 1983; Wagner et al., 1985). Thus, although endothelial cells may be an important antigen presenting cell during the latter stages of an inflammatory episode, they appear to play little or no role in the initiation of CNS lesions in EAE.

1.5.12 *The role of macrophages in EAE*

The induction of EAE appears to require an antigen-processing cell and an immune cell. Some authors have suggested that demyelination occurs when there is intimate contact between inflammatory cells and myelin (Kosunen et al., 1963; Bubis and Luse, 1964; Lampert, 1965) and ultrastructural analysis has shown macrophages apparently "stripping" myelin lamellae from the axon by insertion of processes under the sheath (Lampert and Carpenter, 1965; Lampert, 1967). Activated macrophages secrete phospholipases (Trotter et al., 1982) and proteinases (Cammer et al., 1978) which may result in the breakdown of acidic proteins. MBP and the acidic proteins which are buried in the lipid bi-layer are particularly susceptible to protease activity (Einstein and Czejtey, 1968; Brosnan et al., 1981). Phospholipases may cause the exposure of these proteins to proteolytic attack. In support of this notion, depletion of either donor or recipient macrophages results in the failure of induction of EAE (Panitch and Ciccone, 1981; Brosnan et al., 1981; Killen and Swanborg, 1982a).

1.5.13 *The Role of Antibody in EAE*

The role of antibody in EAE is still unclear although a role for circulating factors has been suggested (Bornstein and Appel, 1961; Brosnan et al., 1983). MBP-specific antibody can be detected in serum by day 6 after antigenic challenge (Gonatas et al., 1974). Serum from recovered animals can protect against active induction of disease if administered during the first 8 days post inoculation (Hughes, 1974) although it will not prevent cellular transfer of disease in rats (Richert et al., 1982). Heat-inactivated sera from rabbits with acute EAE has been demonstrated to induce demyelination in myelinated cultures of rat cerebellum (Bornstein and Appel, 1961) suggesting that anti-myelin antibodies may be responsible for this effect (Bornstein and Raine, 1976; Raine et al., 1978). Although intravenous injection of EAE sera fails to induce CNS damage (Paterson, 1971) possibly due to the BBB, when EAE sera is injected into the lateral ventricles of guinea-pigs demyelination is evident (Jankovic et al., 1965). These studies demonstrate a potential demyelinating activity of EAE sera.

There is no direct evidence suggesting that antibody plays a role in the pathogenesis of EAE. However, B cell and immunoglobulin-deficient rats fail to develop clinical or histological evidence of EAE when sensitized with whole spinal cord or MBP. The T cells were found to be unaltered as they responded normally to PHA and could reject tissue allografts (Willenborg, 1983). The transfer of anti-MBP antibody containing serum to MBP-sensitized Ig deficient rats resulted in the subsequent development of EAE (Willenborg, 1986). Cells producing antibody to MBP have been detected in EAE lesions (Lennon et al., 1972). Treatment of neonatal rats with antiserum to IgM inhibits both

clinical and histological EAE (Willenborg and Prowse, 1983). MBP sensitized Ig deficient rats, which do not develop disease, generate lymphocytes capable of transferring disease to naive rats and also disease develops in these animals after induction with EAE effector cells (Willenborg, 1986). However, the transfer of purified T cells into mice induces disease without detectable antibody production (Bernard et al., 1976). Antibody production doesn't correlate with onset, severity or relapse of EAE (Lisak et al., 1969; Lennon et al., 1971; Tabira and Endoh, 1985) and serum does not transfer disease to normal animals (Kabat et al., 1947; Chase, 1959; Bernard et al., 1976). It is possible that antibody may play a role in demyelination within inflammatory lesions, and in recovery and resistance to re-induction of EAE (Paterson and Harwin, 1963; Willenborg, 1979, 1980).

1.5.14 *The Role of Cytokines in EAE*

Several investigators have claimed to have found a strong correlation between differential cytokine secretion and encephalitogenicity (Powell et al., 1990; Tokuchi et al., 1990) and immunomodulators such as TNF-α, IFN-γ and IL-6 have been implicated in the pathogenesis of demyelinating diseases such as EAE and experimental autoimmune neuritis (EAN) (Gijbels et al., 1990; Hartung et al., 1990; Ruddle et al., 1990; Selmaj et al., 1991; Chung et al.,1991).

1.5.14.1 Gamma interferon

IFN-γ, a lymphokine which has wide ranging effects on many cells is produced mainly by activated T cells (Morris et al., 1982: Sethi et al., 1983). Its primary function in the CNS is thought to be the induction of expression of class II MHC on various cell types. Thus, these IFN-γ responsive cells become active in antigen presentation. In addition, IFN-γ modulates antibody production (Wong et al., 1984). In EAE, systemic (Billiau et al., 1988) and intravenous (Voorthuis et al., 1990) administration of IFN-γ exhibits immunosuppressive properties. Despite this IFN-γ upregulates astrocytic class II MHC expression and superoxide production by microglia, processes relevant to the development of disease (Woodroofe et al., 1989; Voorthuis et al., 1990). Consequently this cytokine is viewed as proinflammatory at the local level (ie. brain and spinal cord), while exerting anti-inflammatory effects systemically (Billiau et al., 1988). Possibly the latter is related to the induction of T suppressor cells, which have been suggested to inactivate T helper subsets (Noma and Dorf, 1985).

1.5.14.2 Tumour Necrosis Factor

The immunopathological significance of TNF-α is unclear. TNF induces IFN-γ and IL-2 secretion (Kohase et al 1986; Naworth et al 1986), enhances T and B cell

proliferation and differentiation (Schenrich et al 1987, Jelinek and Lipsky 1987) and when incubated with IFN-γ, TNF-α enhances astrocyte class II MHC mRNA expression (Vidovic et al., 1990). TNF causes the endothelial cell surface, which is usually an anticoagulant, to become a procoagulant (Bevilagua et al 1986) increasing its adhesiveness for different leukocytes (Bevilacqua et al 1985), alters the immunologic properties of vascular endothelium (Old, 1986) by increasing their permeability (Stern and Naworth, 1986) and MHC expression (Collins et al 1986) and TNF also stimulates rat macrophages and human vascular endothelium to release platelet-activating-factor (PAF) (Camussi et al, 1977) which alters the vascular tone and increases vascular permeability (Camussi, 1986; Bussolini et al, 1987; Camussi et al., 1991). TNF may also cause myelin damage as it has been shown that TNF produces myelin swelling and oligodendrocyte death *in vitro* (Selmaj and Raine 1988).

Chung et al. (1991) demonstrated that EAE resistant (Brown-Norway) rat astrocytes do not express appreciable TNF-α in response to IFN-γ whereas EAE susceptible (Lewis) rat astrocytes do. However, experiments employing neutralising anti-TNF-α antibodies have yielded conflicting results. Actively induced EAE is unaffected by antibody administration (Teuscher et al., 1990), whereas in a murine passive transfer model, symptoms of EAE were markedly reduced (Ruddle et al., 1990; Selmaj et al., 1991). Preincubation of MBP-sensitised T cells with anti-TNF-α antibodies *in vitro* prior to injection had no effect on the ability of these cells to transfer EAE (Selmaj et al., 1991).

1.5.14.3 Interleukins

IL-1 may initiate or promote inflammation within the CNS. IL-1 causes the endothelial cell surface to become procoagulant and suppresses cell surface anti-coagulant activity (Nawroth et al., 1986). Treatment of cultured human endothelial cells with IL-1 results in a concomitant decrease in the production of tissue plasminogen activator (t-PA), the moiety responsible for endogenous thrombolysis and an increase in plasminogen activator inhibitor by suppressing net fibrinolytic activity (Bevilacqua et al., 1986). Treatment with IL-1-α for 15 days following immunisation with MBP+CFA resulted in a longer duration and greater severity of disease with increased weight loss in Lewis rats. An IL-1 antagonist significantly delayed the onset of EAE, reduced the severity of paralysis and weight loss and shortened the duration of disease (Jacobs et al., 1991). In related studies, local elevations in IL-6 correlated with disease severity in mice (Gijbels et al., 1990) and treatment of Lewis rats with anti-IL-2 receptor antibody protected recipients from passive transfer of EAE (Engelhardt et al., 1989).

In summary, both TNF-α and IFN-γ have paradoxical effects on autoaggressive processes depending on the administration schedule and the experimental system. During

an immune response, the production of cytokines is significantly increased and may play a significant role in the pathophysiology of disease.

1.5.15 *Cause of clinical symptoms*

The correlation between the number of lesions in the CNS and flaccid paralysis is poor (Hoffman et al., 1973; Raine, 1980; Simmons et al., 1982, 1984) and other mechanisms have been proposed to explain the loss of nerve transmission which results in paralysis. Slowing of internodal conduction or conduction block of selected fibers (Pender and Sears, 1982, 1984; Pender, 1986; Raine, 1976; Arnon, 1981) and the extent of oedema in the spinal cord (Leibowitz and Kennedy, 1972; Levine et al., 1966; Simmons et al., 1982) have been suggested as mechanisms of dysfunction .

One of the features of the clinical progression of EAE is the ascending nature of weakness and sensory loss (Williams and Moore, 1973; Simmons et al., 1982). Ascending impairment of tail nociception occurs in both MBP-induced EAE and in whole spinal cord-induced EAE (Pender, 1986b). This can be accounted for by the caudally increasing length of the spinal roots (Waibl, 1973) which increases the probability of lesion formation progressively in a caudal direction. Thus, the probability of demyelination (Pender, 1986b) or oedema (Simmons et al., 1982) induced conduction block increases caudally and can anatomically account for the ascending weakness of EAE. Daniel et al. (1983) found that permeability to mannitol was evident in the lower spinal cord of rats developing EAE. This permeability reached its highest level during the acute phase of disease and slowly returned to normal from 15 days post inoculation starting from the caudal end of the spinal cord. Pender (1986b) further suggests that caudal roots show more extensive demyelination.

1.5.15.1 Oedema

Clinical signs correlate best with the extent of oedematous changes in the spinal cord (Blakemore et al., 1989). It has been suggested that BBB permeability changes which result in oedema are responsible for clinical symptoms (Leibowitz and Kennedy, 1972; Daniel, 1981; Simmons et al., 1981, 1982, 1984; Kerlero de Rosbo et al., 1985). Vascular permeability changes are evident in the rat by day 6 post immunisation (Oldstone and Dixon, 1968; Daniel et al., 1981 and 1983; Suckling et al., 1983; Sobel et al., 1984). However, CFA alone can cause increases in vascular permeability (Suckling, 1984). Lymphocyte migration and vascular permeability are not interdependent as vascular permeability is a serotonin-dependent phenomenon (Schwartz et al., 1977) whereas lymphocyte migration is not (Rose and Parrot, 1977). Inflammatory cells may contribute to BBB dysfunction and associated oedema formation in EAE (Claudio et al., 1990).

Hawkins et al. (1991) found that BBB permeability as measured by MRI and gadolinium leakage was associated with duration of clinical relapse in CREAE.

There is considerable swelling of the spinal cord during EAE (Levine et al., 1966) and oedema associated with fibrin deposition in EAE lesions has been implicated in the loss of conduction of impulses down nerve fibres (Paterson, 1976; Kristensen and Wisniewski, 1977). Effector lymphocytes enter the CNS, recognise MBP in a class II MHC restricted way (Steinman et al., 1981). The recognition results in the release of lymphokines which attract monocytes and macrophages to the CNS. This accumulation of inflammatory cells initiates oedema formation. The oedema is associated with fibrin deposition in the lesions (Paterson, 1976) and results in a loss of conduction of nerve impulses. More recently, oedema has also been proposed as the mechanism for neurological signs in passively transferred acute EAE (Sedgewick et al., 1987) and CREAE (Butter et al., 1989).

1.5.15.2 Fibrin deposition

Fibrin is deposited in lesions of EAE (Oldstone and Dixon, 1968; Paterson, 1976; Rauch et al., 1978) and has been suggested as a cause of paralysis in EAE (Paterson, 1976). Fibrinogen-depleted rats do not develop paralysis although the extent of cellular infiltration is the same as controls (Paterson, 1976). Vascular permeability allows extravasation of plasma proteins into the perivascular space where fibrinogen is converted to insoluble fibrin and deposited (Colvin and Dvorak, 1975). Neural cells are also a rich source of thromboplastin and any damage in the CNS can lead to the release of thromboplastin and additional fibrin formation (Graebar and Stuart, 1978). In histological sections of spinal cord from Lewis rats with acute EAE, Ackerman et al. (1981) found fibrinogen was restricted to vessels containing cellular infiltrates suggesting that fibrin deposition does not occur as a result of general vascular permeability, leading to plasma protein extravasation, but is produced by infiltrating cells. As fibrinogen is a large molecule (340,000 daltons) it can possibly only enter lesions when vascular damage has occurred. Although the fibrin deposition in EAE has been attributed to vascular leakage, the possibility exists that neural cells are the source of inducible procoagulant activity and may contribute to fibrin deposition in EAE lesions. However, treatment with two platelet-activating factor antagonists had no effect on the development of disease (Velna et al., 1991).

1.5.15.3 Demyelination

There is evidence to suggest that it is demyelination which produces the clinical signs of EAE (Pender, 1987; Pender et al., 1990). Demyelination causes conduction block with refractory periods of transmission (McDonald and Sears, 1970; Smith et al.,

1979,1981; Bostock and Grafe, 1985; Kaji et al., 1988). Motor conduction abnormalities have been found in the region of the spinal cord ventral roots in acute EAE in Lewis rats (Pender, 1988b). The clinical form and amount of demyelination are different among species and antigen used. Guinea-pigs show the greatest amount of demyelination and the least axonal damage. More demyelination is seen in animals with a more chronic disease process (Tabira and Sakai, 1987). Chronic relapsing EAE, like MS, is characterised by extensive demyelination associated with lesions. Guy et al. (1991) measured myelin sheath thickness and axon diameter of optic nerves in guinea-pigs suffering from EAE. Both myelin sheath thickness and axonal diameter of animals with EAE were significantly lower than normal animals. Jones et al. (1990) found that prominant perivascular demyelination associated with mononuclear inflammation was evident in passively induced EAE in Buffalo rats and that demyelination occurred without the addition of demyelinating antibodies or subsequent clinical relapses. In contrast, there is very little demyelination in EAE which is MBP-induced (Lampert, 1965). Although MBP is necessary for the induction of the inflammatory response, demyelination may be caused by an additional immune response against other lipid myelin surface antigens (Moore et al., 1984). Demyelination occurs in cerebellar cultures if EAE sera from animals immunised with whole CNS tissue is used (Bornstein and Appel, 1961) but not if MBP as the immunogen is used (Seil et al., 1968). Some authors have suggested that demyelination occurs when there is intimate contact between inflammatory cells and myelin (Kosunen et al., 1963; Bubis and Luse, 1964; Lampert, 1965) and ultrastructural analysis has shown macrophages apparently "stripping" myelin lamellae from the axon by the insertion of processes under the sheath (Lampert and Carpenter, 1965; Lampert, 1967). However, others claim that demyelination cannot be the cause of neurological signs of acute EAE as demyelination is reported to be sparse or absent in these models, particularly if MBP is the inoculum (Hoffman et al., 1973; Lassman and Wisniewski, 1979; Panitch and Ciccone, 1981; Raine et al., 1981; Simmons et al., 1981, 1983; White, 1984; Kerlero de Rosbo et al., 1985) and that recovery occurs too rapidly to be attributed to remyelination (Panitch and Ciccone, 1981; Simmons et al., 1981).

1.5.15.4 Astrocytic gliosis

Astrocytes form scar tissue in response to injury to the nervous system (reactive gliosis) (Miller et al., 1986) and may be involved in the pathogenesis of EAE. Fibrillary astrogliosis is one of the preclinical changes evident in EAE (Field, 1961; Bubis and Luse, 1964; Lampert, 1967). Gliosis is a prominant feature in the chronic model of EAE in the guinea-pig and mouse (Linington et al., 1984; Raine, 1983; Smith et al., 1984, 1985). Smith et al. (1983) have shown that in acute EAE in the Lewis rat enhanced immunocytochemical staining of GFAP is evident 10 to 12 days post inoculation. The

intensity of staining increased with time post inoculation. Reactive astrocytes were distributed throughout the tissue and persisted until clinically evident disease had subsided, with the increased staining correlating with an increased permeability of the BBB. Cammer et al. (1990) found the increased GFAP staining evident in the spinal cord in acute EAE in Lewis rats occurred primarily in the white matter tracts and was not restricted to areas of inflammation. Goldmuntz et al. (1986) also showed increased staining for GFAP of Lewis rats with acute EAE commencing 10 days post inoculation and increasing with time. These reults suggest that astrocytes may play an important role in the pathogenesis of EAE.

In a study on CREAE in the SJL/J mouse, Smith et al. (1988) found astrocytic hypertrophy near demyelinating lesions which eventually resulted in the formation of gliotic plaques in the affected mouse. Astrocytes may perpetuate myelinolysis within the lesion as they contain lysosomal enzymes which are activated in EAE lesions and are capable of degrading myelin (Arstila et al., 1973; Allen, 1983). Demyelinated axons are associated with astrocytic processes (Black et al., 1987) and gliosis has been shown to inhibit remyelination *in vitro* (Raine and Bornstein, 1970) and impede regenerating axons in the dorsal roots of the spinal cord (Liuzzi and Lasek, 1987). Clinical and histological EAE can also be induced in guinea-pigs and monkeys by the injection of human glioblastoma, suggesting that glial cells are associated with the symptoms of EAE (Bigner et al., 1981). Thus, astrocytic gliosis may contribute to the clinical symptoms of EAE, particularly if demyelination is a major cause of disease symptoms.

1.5.15.5 Axonal degeneration

Axonal damage and degeneration is probably an important pathophysiological mechanism in CREAE (White et al., 1989; Pender et al., 1990; Stanley and Pender, 1991), HEAE (Lampert, 1967; Hansen and Pender, 1989) and acute EAE in rats (White and Bowker, 1988) and mice (Tabira and Sakai, 1987). During the acute paralytic phase of EAE in rats, large numbers of the bulbospinal axons that contain the monoamine neurotransmitters, serotonin and noradrenalin appear to be damaged (White et al., 1985). Axonal dysfunction can also occur as a result of the action of inflammatory mediators such as lymphokines without detectable morphological changes (Arezzo et al., 1988). White et al. (1990) found that axonal damage was equally pronounced whether EAE is induced by MBP or whole spinal cord homogenate which suggests that the damage is not a result of immune attack directed against monoaminergic or peptidergic antigens present in the inoculum. Axonal degeneration probably contributes to persistent neurological dysfunction of CREAE and HEAE.

1.5.15.6 Neurotransmitters

Neurological signs of EAE have been attributed to impairment of monoaminergic neurotransmission (Carnegie, 1971; White, 1984). Interference with neurotransmission due to decreases in noradrenaline and serotonin in the lumbar-sacral region (Lycke and Roos, 1973; Honeggar and Isler, 1984), an area rich in serotonin terminals, may account for the localisation of clinical symptoms of EAE (Lennon and Carnegie, 1971). Guinea-pigs showing symptoms of EAE show depletion of receptors (Weinstock et al., 1977; White, 1979). MBP binds serotonin (Weinstock et al., 1977) and this may be the cause of the decreased levels in EAE. However there are increased levels of monoamine oxidase activity in guinea-pigs with EAE (Saregea et al., 1965), reflecting increased levels of serotonin, and this may partially account for the reduction in serotonin levels.

Rabbits injected with neural tissue in adjuvant have shown disturbances of neuro-electrical transmission (Feldman et al., 1969). The MBP antigen has also been found to cause a long-lasting depolarisation of neurones leading to a loss of transmission in frog spinal cord (Honeggar et al., 1977; Gahwiler and Honeggar, 1979; Isler and Honeggar, 1983) and the intraneural electrical activity of mammalian brain cultures is blocked by the addition of sera from animals with EAE or patients with MS (Ross and Bornstein, 1969). There is considerable depletion of serotonin and noradrenaline in the lumbo-sacral region of the spinal cord during clinical episodes and recovery phases in Lewis rats with CREAE (Krenger et al., 1986).

1.5.15.7 Oxidative damage

The action of oxygen radicals can result in substantial damage in the nervous system (reviewed by Halliwell and Gutteridge, 1985a). Oxidative damage of the CNS is suggested to be one of the mechanisms underlying the pathogenesis of EAE. Kassabova et al. (1990) found oxidative damage in the lumbar spinal cord of guinea-pigs with clinical signs of EAE, whereas injection of animals with vitamins C and B2 during the disease latent period suppressed EAE development. Disruption of the lipid layer by the products of superoxide or hydrogen peroxide could expose MBP and proteolipid protein to proteolytic attack with demyelination reflecting a combination of oxidative damage by macrophages and a protein generated immune response. Damage to myelin appears to result from contact with macrophages (Lampert, 1968) and macrophages activated by lymphokine produce superoxide and hydrogen peroxide (Freund and Pick, 1985). Another explanation for the loss of serotonin is that free radicals are scavenged by serotonin. Possibly lymphoid cells liberating superoxide or hydrogen peroxide could cause initial damage to myelin membrane in EAE and exposure of myelin proteins may perpetuate the specific immune rsponse.

1.5.16 *Recovery from disease*

The immunoregulatory events determining recovery and subsequent resistence have not been clearly defined. It seems most likely that the resolution of oedema is the initial factor contributing to the rapid recovery of disease; whereas longterm changes may involve remyelination, axonal regeneration and synaptic re-organisation.

1.5.16.1 Resolution of oedema

McDonald (1974) suggested that the dispersal of oedema in optic neuritis in humans may allow conduction to return to demyelinated fibres. The study by Jacobson et al. (1979) on optic pathways of cat demyelinated by diphtheria toxin injection supported this hypothesis. Early recovery was suggested to be due to the dispersal of oedema at the site of the lesion, with later recovery involving more complex re-organisation of synapses (Jacobson et al., 1979). However, resolution of axonal dysfunction caused by the toxin could also result in early recovery.

1.5.16.2 Remyelination

Remyelination is evident in EAE (Lampert, 1965; Wisniewski et al., 1969; Lassman et al., 1980b) although it has generally been considered to be a late feature of demyelinated chronic lesions (Prineas et al., 1969). In contrast, Lassman et al. (1980b) demonstrated that the capacity for remyelination in the CNS differs at various stages of disease. They suggested that the decrease in remyelination in later stages of disease is due to a more destructive demyelination occuring at later stages, the unavailability of oligodendrocytes, and to the pronounced astrocytic gliosis which possibly affects remyelination.

As remyelination occurs, conduction is restored resulting in a return to transmission (Smith et al., 1979,1981). Koles and Rasminsky (1972) found that as little as 3% of normal myelin thickness could be sufficient to ensure saltatory conduction. Pender (1988) performed histological and electrophysiological studies and found that the functional recovery from acute EAE in Lewis rats was due to the remyelination achieved by Schwann cells in the PNS and oligodendrocytes in the CNS. Shrager (1988) also found by measuring ionic currents in remyelinating frog nerve fibres, that conduction can be improved by remyelination via the aggregation of existing sodium channels at new nodes of Ranvier. Can clinical recovery be due to the repair of the myelin sheath? Raine and Traugott (1983) found that animals treated with a combination of MBP and galactocerebroside during chronic relapsing EAE displayed widespread remyelination and oligodendroglial proliferation. On the other hand, some researchers believe that remyelination occurs too slowly in demyelinating lesions to contribute effectively to clinical recovery (McDonald, 1974).

1.5.16.3 Axonal regeneration

Recent studies suggest that even after axonal regeneration, abnormal physiological properties still persist in synaptic connections (Kierstead et al., 1989) and conduction velocities (Cragg and Thomas, 1964a; Feasby et al., 1981; Bowe et al., 1989). With increasing axonal diameter and myelin sheath thickness, conduction velocities do improve but may never return to normal values (Cragg and Thomas, 1964a). These persistent changes would limit recovery and more possibly contribute to neurological deficit.

1.5.16.4 Neuroendocrine-mediated immunoregulation

Several investigators have suggested that the production of cytokines during an immune response induces endocrine-mediated suppression of the immune response (Munck et al., 1984; Besedovsky et al., 1986; Salpolsky et al., 1987; Kroemer et al., 1988) and neuroendocrine-mediated immunoregulation could be relevant in the recovery phase of EAE. Exogenous stress has been found to suppress EAE in Lewis rats (Levine et al., 1962) and Mason et al. (1990) have proposed that the resistance to EAE seen in some strains is due to corticosterone-mediated suppression. In the susceptible Lewis strain, recovery from paralysis correlates with increased levels of corticosterone. If susceptible Lewis rats are adrenalectomised, they are unable to recover and the disease becomes uniformly fatal, an effect which can be reversed by steroid replacement therapy (Mac Phee et al., 1989). The critical time for adrenalectomy was found to be 24 to 48 hours before paralysis occurred (Mason et al., 1990). Adrenalectomy performed 12 to 14 days post inoculation does not result in the development of disease suggesting that the long-term refractory period found in recovered rats does not depend on corticosterone-mediated suppression (MacPhee et al., 1989). The PVG strain which is not susceptible to EAE, develops severe disease after adrenalectomy with steroid replacement therapy preventing the fatal progression of disease. PVG rats were also found to have significantly higher basal levels of corticosterone than those in Lewis rats and to produce a more vigorous response to stress (Mason et al., 1990). These results suggest that possibly an exaggerated adrenal response may result in recovery from disease but not the long-term resistance to active reinduction.

1.5.17 *Resistance to reinduction of disease*

In addition to recovery, animals become resistant to further attempts of active induction of disease (Vandenbark and Hinrichs, 1974). The events determining this subsequent resistance are unknown. The immune system becomes tolerant of the body's molecules because mechanisms either suppress (suppression), eliminate self reactive cells

(deletion) or turn them off (anergy). Although resistant to active induction of EAE, animals remain fully susceptible to the passive transfer of disease (Willenborg, 1979) suggesting that an active suppressor mechanism is either overwhelmed by the number of effector cells injected with passive transfer or suppressive mechanisms act at an early stage of disease development before T cells are primed to neural antigen (Hinrichs, 1984). Suppression was also demonstrated to be expressed systemically rather than just locally as resistance to reinduction is independent of the site of primary and sensory sensitisation (Willenborg, 1981). Whatever the mechanism of resistence, total body irradiation (Willenborg, 1982), pertussigen (Waxman et al.,1982) and cyclophosphamide (Miyazaki et al., 1985) allow reinduction of disease in convalescent rats.

1.5.18 *Inhibition and Treatment of EAE*

1.5.18.1 Hormonal regulation of EAE

Steroids have been shown to have an immunosuppressive effect on EAE (reviewed by Komarek and Dietrich, 1971). ACTH and glucococorticosteroids, which includes both endogenously produced steroids (eg. cortisol or hydrocortisone) and the more potent synthetic analogues such as prednisone, prednisolone, methylprednisolone and dexamethasone, are the more commonly used immunosuppressive agents (Mertin, 1985). High methylprednisolone dose given prior to EAE induction, significantly increases disease duration. In contrast, when given after the onset of clinical disease this drug has a marked beneficial effect (Steiner et al., 1991). Steroids act at a variety of stages in the immuno-inflammatory cascade. For instance, they cause an inhibition of the T cell growth factor IL-2 (Pinkston, 1987), prevention of neutrophil chemotaxis (Cupps and Fauchi, 1982), lowering of monocyte superoxide production (Bell, 1986) and arachidonic acid release (Flower, 1984) and diminished local antibody production resulting from inhibition of granuloma formation by cortisone (Kabat et al., 1952). In addition, MacPhee et al. (1989) reported that adrenalectomised rats with EAE were unable to recover from an acute episode of the disease. Steroid replacement therapy prevented death in these rats. Unfortunately, there are a number of adverse side effects that can be attributed to the use of steroids, including renal insufficiency, peptic ulceration, osteoporosis and hyperglycaemia (Marble et al., 1980) and the possibility of increased susceptibility to infections as the bactericidal and antimicrobial capacities of the immune system are compromised.

1.5.18.2 Irradiation

Radiation has been reported to have varying effects on the induction of EAE (Levine et al., 1969) with variations probably being related to the species studied, the dose, the timing and the type of radiation. Some investigators found that irradiation of recipients completely inhibited cellular transfer of inflammatory disease (Levine et al., 1969; Werdelin and McCluskey, 1971) and when recipients were restored with bone marrow cells inflammation developed (Werdelin and McCluskey, 1971), whereas others found low dose irradiation (350rads) facilitated induction of EAE with MBP-reactive T cell lines (Zamvil et al., 1985). Sedgewick et al. (1987) reported that regardless of the status of the recipient (irradiated or not), clinical signs were induced when animals received MBP-reactive cells although the number of infiltrating leukocytes was reduced in irradiated animals. The augmenting influence of irradiation on EAE could be due to irradiation increasing the permeability of the CNS and the interaction of sensitised cells or antibody with CNS antigen.

1.5.18.3 Cyclosporin-A

Cyclosporin-A (CsA) is effective in inhibiting EAE and rejection of tissue grafts as well as other models of immunologically mediated disease. A daily oral dose of 15mg/kg or more prevents the induction of the disease in Lewis rats and histological analysis demonstrates that the average number of inflammatory lesions in the CNS is substantially reduced (reviewed by Nussenblatt et al., 1986). The immunosuppressive effect of CsA is possibly related to its capacity to inhibit the sensitisation of T cells to specific antigen (Klaus and Chisholm, 1986; Benson et al., 1989). CsA inhibits the expression of MHC antigens (Autenried et al., 1985), T cell proliferation (Kumagai et al., 1988) and IL-2 and IFN-γ expression (Kronke et al., 1984; Granelli-Piperno, 1990; Kroemer et al., 1990). However, low dose CsA administered immediately after immunisation until day 22 post immunisation does not prevent a first attack of EAE (Polman et al., 1988). CsA in fact contributes to the pathology of EAE, by inducing clinical relapses in 100% of Lewis rats examined, with the relapses in general being clinically severe and long-lasting or even continuous. Pender et al. (1990) investigated the neuropathology of CREAE induced by inoculating female Lewis rats with guinea-pig spinal cord and adjuvants and treated with low dose CsA and found that severity and chronicity of neurological signs were increased. These results are consistent with the known inhibitory effect of CsA on the initial steps in the sensitisation of cells to specific antigen and on T cell proliferation but not progression (Kumagai et al., 1988).

1.5.18.4 Inhibitory effect of neural tissue homogenates

It was first observed by Ferraro and Cazzulo (1949) that repeated injections of nervous tissue homogenates interfered with the development of EAE. Many researchers have subsequently found that injections with relatively large amounts of CNS tissue diminish or abolish development of disease (Paterson, 1958; Waksman, 1959; Kornblum, 1968) and it is thought that these large doses inhibit immunologic responses to CNS tissue or antigen in adjuvant. Oral administration of MBP also suppresses EAE in Lewis rats immunised with MBP in adjuvant with antibody responses to MBP also being suppressed (Khoury et al., 1990).

1.5.18.5 T cell vaccination

If rats were inoculated with subencephalitogenic doses of activated MBP-reactive cells, animals not only did not develop disease but were protected from subsequent EAE induction (Beraud et al., 1989). MBP-reactive clones, rendered harmless by chemicals or radiation, and reinjected into animals also suppress EAE, possibly by sensitising the animals to the myelin-destroying T cells (Cohen, 1988). Whether this approach is viable as a general therapy for autoreactive T cells requires further investigation.

1.5.18.6 Sulphated polysaccharides in EAE

Heparin and fucoidan, two anti-coagulants, are potent inhibitors of passively induced EAE (Willenborg and Parish, 1988). Clinical signs are abrogated, but with heparin there are no inflammatory lesions seen in numerous histologic sections taken from treated rats. Heparin and fucoidan have been shown to stop lymphocytes from recirculating (Brenan and Parish, 1986) causing a three-fold and six-fold increase in blood lymphocytes, respectively. This result suggests that the inflammatory cells have failed to enter the CNS. Heparin also inhibits actively induced disease (Lider et al., 1989). Rats usually develop disease after 10 to 11 days but when given an initial dose of heparin disease is delayed by 6 days. Heparin and possibly fucoidan may inhibit disease by blocking the migration of activated lymphocytes by binding to heparanases on the surface of activated lymphocytes as non-cleaveable substrates that occupy and block the active site of the enzyme. Chemically modified heparins lacking anti-coagulant activity are anti-inflammatory and this correlates with the ability of the modified heparins to inhibit heparanase. As sulphated polysaccharides are inhibitors of inflammation in the CNS, these compounds may be used as potent, non-toxic anti-inflammatory agent useful in the treatment of inflammation of the CNS. Furthermore, the lack of toxicity may enable them to be used chronically to prevent further exacerbations.

Finally, it should be noted that agents effective in suppressing EAE, ie. anti-lymphocyte serum, MBP (Eylar et al., 1972; Levine et al., 1972; Driscoll et al., 1974;

Raine et al., 1977), 6-mercaptopurine, glucocorticosteroids, phytohaemagglutinin (reviewed by Arnason, 1972), linoleic acid (Meade et al., 1978) and proteinase inhibitors (Smith, 1980), have not been successful in treating MS.

1.5.19 *Age Related Factors in EAE*

EAE is an age dependent disease which requires the presence of a mature lymphoid system (Paterson et al., 1970). Neonatal rats are resistent to EAE and become susceptible at approximately 8 weeks of age. The encephalitogenic activity of the CNS material parallels the ontogenic development of myelin, which is poorly developed at birth, reaching sufficient quantities by 2 weeks of age to induce EAE in guinea-pigs (Schwenkter and Rivers, 1934). CREAE in GPs is most readily achieved at weaning. Earlier animals lack effective T cell response to produce disease. Later EAE tends to be monophasic (Arnon, 1981). Probably weaning (and adolescence in humans) is associated with maturation of the system of suppressor cells and immune regulation (Waksman, 1988).

Neonatal rats are apparently resistant to passive induction of EAE as they do not show clinical or histopathological evidence of disease after the transfer of cells. However, these rats are thought to be asymptomatic carriers of the disease, as at 10 to 12 weeks of age, they can be actively sensitised with MBP in CFA and they will develop disease significantly earlier than control animals (7 to 8 days compared with 10 to 11 days). The earlier onset of disease in rats that are neonatally induced is thought to be a memory response due to the persistence of transferred cells. Animals not only develop disease earlier but MBP antibody levels increase at Day 5 rather than Day 7. These rats might appropriately be considered asymptomatic carriers of the autoimmune disease. An early etiological event may set up an autoimmune carrier state and the MS patient may in effect be a reservoir of autoimmune effector cells which are periodically activated by one or more unknown stimuli.

With advancing age the lymphoid system undergoes atrophy and has a decreased ability to mount a T cell immune response (Walford, 1969; Roberts-Thomson et al., 1974). Levine and Sowinski (1976) demonstrated an age related decline in susceptibility to EAE. They found that with intense immunisation (MBP+CFA+pertussis vaccine) of rats (inbred Fischer 344 males) aged 2, 6 and 12 months, EAE developed at a similar rate and severity. There was a lesser response in 18 and 24 month olds with a more protracted incubation period and milder or absent signs. However, with less intense immunisation (without pertussis or 1μg MBP) almost all 6 month olds developed disease but not a single 24 month old developed EAE under these conditions. In their passive transfer model, Levine and Sowinski (1976) found that only 2 out of 4 of the 24 month olds developed EAE. The data indicated an age related decline in susceptibility to EAE although histologic examination of inoculated feet and

draining lymph nodes revealed no deficiencies in the processing of the antigenic inoculum. However, none of their rats seemed to be free of disease and extensive leukemic infiltration of lymphoid organs might have immunosuppressed recipients. Due to the array of pathologic lesions found in aged F344 rats (Sass et al., 1975; Levine and Sowinski, 1976), it was suggested that this was not a good model for assessing age related factors and possibly a strain with a longer life-span would be a better model. In old mice, susceptibility to EAE induced with bovine proteolipid apoprotein (PLP) and MBP is reduced significantly although some old mice develop histologic EAE without clinical signs. Lymphocyte proliferative response to mitogens and antigens, and IL-2 production were also decreased in aged mice (Endoh et al., 1990). It was subsequently found that the reduced susceptibility of old mice to EAE and the decreased T cell functions cannot be restored by the treatment of old mice with thymic hormones (Endoh and Tabira, 1990). In contrast, Mc Farlin et al. (1974) reported recurrent EAE in aged (6 months old) female Lewis rats, whereas young rats of the same strain exhibited a monophasic course of disease and Ben-Nun et al. (1981) found Lewis rats developed recurrent or chronic EAE when sensitised with MBP at an advanced age.

1.5.20 *EAE as an experimental model*

MS and EAE are both diseases characterised by considerable infiltration of leukocytes into the CNS. As alterations in the ratios of T cell subsets during clinical relapses are evident (Reinherz et al., 1980; Brinkman et al., 1983; Weiner and Hauser, 1983; Antel et al., 1984) much research has focused on immune regulation in EAE and MS. Similar to MS, it is the myelin sheath which is destroyed with the axon being spared. For this reason it was hypothesised that the "antigen" for EAE and MS was residing in myelin.

However, there are clinical, pathological and immunological differences between acute EAE and MS (Adams, 1959; Field, 1967; Paterson; 1971; Raine; 1976) resulting in major criticisms of its use as a model for MS. Acute EAE has a monophasic disease with a high mortality rate, whereas MS usually has a chronic relapsing or chronic progressive course. In acute EAE, the lesions are of the same age and demyelination and gliosis are often absent whereas in MS there are both old and recent lesions, extensive demyelination and gliosis. Pender (1988) makes the point that one possible major difference between EAE and MS is that the neurological signs of EAE may be attributed to peripheral nervous system (PNS) involvement whereas MS is diagnosed on the presence of neurological signs in the CNS. PNS involvement has been described in EAE in the rabbit (Waksman and Adams, 1955; Wisniewski et al., 1969), mouse (Waksman and Adams, 1956), guinea-pig, rats and mice with chronic relapsing EAE (Madrid and Wisniewski, 1978; Lassman et al., 1980; Brown et al., 1982) and the monkey (Ferraro and Roizin, 1954).

Acute EAE is considered to be a satisfactory model for the human disease acute disseminated encephalomyelitis (Adams, 1959; Levine, 1971; Lisak et al., 1974) whereas chronic relapsing forms of EAE are a more appropriate model for MS. Chronic relapsing forms of EAE in the immature strain 13 and Hartley guinea-pigs are clinicopathologically similar to MS. Both diseases have a relapsing clinical course, inflammatory lesions, primary perivenous demyelinated plaques, gliosis, loss of oligodendrocytes with some axonal loss within the plaques in the later stages of disease (Lassman and Wisniewski, 1979). Although there are dissimilarities between MS and EAE, EAE is a useful model to study neuroimmunological relationships in the CNS. Abberations in immune regulation may be a result of disease rather than a cause and it is important to establish the site and nature of the lesions responsible for neurological signs.

1.6 Aims of the present study

1.6.1 *T cell entry into the CNS*

Lymphocytes are normally found in low numbers in the CNS. Migration of T cells to the CNS and their cellular distribution in EAE lesions is well documented. How their entry is achieved during EAE in the absence of gross damage to the vascular endothelium is unknown. This study addresses the hypothesis that "activated" lymphocytes of any specificity enter the CNS but only those with neuro-antigen specificity persist and cause pathology.

The host contribution to passively induced EAE was also examined by assessing whether the entrance and accumulation of neurospecific cells was altered by irradiation of the recipients.

1.6.2 *Age related changes in EAE*

The development of EAE requires an intact lymphoid system and with advancing age the lymphoid system undergoes atrophy and has a decreased ability to mount a T cell immune response. MS and its animal model EAE are both age dependent diseases. It is rare for children to develop MS. Similarly, neonatal rats are resistant to EAE and become susceptible at approximately 8 weeks of age. Lesions of MS begin in relation to small blood vessels and alterations of the clotting mechanism have been reported in MS patients and in ageing. Lack of the endothelial smooth muscle cell relaxing hormone (nitric oxide) seems to be involved in the occurrence of spasm of blood vessels and differences in the production of this hormone may possibly be involved in the pathogenesis of MS and EAE. Previous research also suggests that aberrations in immunoendocrinological communication may be the cause of immune dysfunction in both autoimmunity and ageing, corticosterone playing an important role in the recovery of animals from EAE.

The immunoregulation of EAE was investigated by specifically looking at age related differences and testing for differences in immunoregulatory factors such as the production of cytokines and the effects of hormone levels on the disease state.

A combined clinical, biochemical and histopathological study of acute EAE in Lewis(JC) rats of differing ages was performed to understand the clinical manifestations of this neurological disease. The specific aims of the study were:

1) to ascertain the functional significance of antigen specificity and state of activation of lymphocytes in entry and accumulation in the CNS;

2) to establish clinical and histopathological changes in disease of aged animals and to determine the mechanisms of recovery;

3) to correlate the clinical, histological changes and recovery with the production of corticosteroids, TNF, nitric oxide, prostaglandins and MBP antibody.

The study of the principles of the inflammatory process within the CNS may lead to pharmacologic or immunoregulatory ways of manipulating the inflammatory process.

CHAPTER 2: MATERIALS AND METHODS

2.1 Animals

Lewis (JC) rats were bred under specific pathogen free conditions at the Animal Breeding Establishment, John Curtin School of Medical Research (JCSMR), Canberra. Lewis (JC) rats bred at the JCSMR carry the AA allelle whereas most other Lewis rats are aa. This strain is fully susceptible to EAE although the disease is not as severe and deaths are infrequent. In lymphocyte labelling studies, only male rats between the ages of 9 and 12 weeks were used as recipients of cell populations. In all experiments, control and experimental animals were age and sex matched. Sterilisation of females by ovariectomy was performed at four days postnatally by Dr. Andrew Hughes, a veterinarian.

2.2 Irradiation

Rats were given total body irradiation with a 100Ci ^{60}Co gamma source one day before receiving cultured cells. Groups of four rats were placed in plastic containers on a revolving stage 15 cms from the source. The dose rate was 60 rads/min with 1000 rads being the total dose delivered. Peripheral blood leukocyte levels were assessed using white cell counting fluid.

2.3 Antigens

MBP was isolated from frozen guinea pig CNS tissue using the method of Deibler et al. (1972). The entire procedure was carried out at 4°C. Spinal cords and brains were weighed and then homogenised in 19 volumes of chloroform/methanol (2:1, v/v) and stirred overnight. The mixture was filtered (Whatman filter paper No. 1) by vacuum and the filtrate discarded. The tissue residue was resuspended in 10 volumes of acetone, stirred for 15 mins and filtered again. The acetone-treated residue was resuspended in 20 volumes ddH_2O (pH 2.1) and stirred overnight. After vacuum filtration (Whatman filter paper No. 54) the residue was resuspend in 5 volumes (ddH_2O). The stirred H_2O suspension was slowly adjusted to pH 3.0 by dropwise addition of 1N HCL and stirred for 1h with the pH being maintained at 3.0, filtered again and the residue discarded. 33.3mls purified 8M urea per 100mls of the pH3 extract was added, and stirred for 10 mins.

Purified MBP was then obtained by ion-exchange chromatography on CM-52 resin (Carboxymethylcellulose, Whatman). Equal volumes of CM-52 to the original weight of spinal cord was added and by dropwise addition of 1N NaOH, the suspension was adjusted to pH 11.6 and stirred for 30 mins. The alkaline suspension was vacuum filtered (Whatman filter paper No. 54). The solid was resuspended in 2 volumes 2M urea - 0.02M NaCL - 0.2 M sodium glycinate solution (pH 11.6), stirred and vacuum filtered. This step was repeated with the same volume of urea-salt-buffer solution and 4 volumes of H_2O was added, stirred

and filtered (repeated twice). The residue was resuspended in 2 volumes H_2O (pH 2.5) with 1 M HCl, stirred for 5 mins and filtered. Resuspended in 2 volumes 0.1 M HCl and stirred for 10 mins. After filtering, the filtrate was dialysed for 2 days in ddH_2O before being freeze dried (Dynavac Model FD12, Australia). The yield of MBP from 50g of original spinal cord material was approximately 50mg. The purified MBP was stored at -4°C.

Grade V ovalbumin (OA) with an electrophoretic purity of approximately 99% was used (Sigma Chemical Co., St. Louis, MO, USA). Purified protein derivative of tuberculin (PPD) was obtained from CSL, Parkville, Vic, Australia.

2.4 Blastogenesis assay

The cross reactivity of MBP- and PPD-specific cell lines was assessed using a lymphocyte proliferative assay. MBP- and PPD-specific cell lines were cultured in quadruplicate in 96-well Linbro culture plates (Linbro Chemical Co., New Haven, Conn.) at concentrations of 10^5/ml or 10^6/ml in 0.2ml RPMI (Flow, McLean, VA) containing 5% foetal calf serum (FCS; Flow Labs. Stanmore, NSW, Australia), 2mM L-glutamine, $5x10^{-5}$M 2-mercaptoethanol (2ME), antibiotics and antigens (either MBP or PPD at concentrations of 25µg/ml and 50µg/ml). Irradiated (2000rads) normal thymocytes were used as accessory cells ($5x10^5$ or $1x10^6$) and the plates were incubated for 72h at 37°C in an atmosphere of 10% CO_2, 7% O_2 with the balance N_2. Five hours before harvesting, cultures were pulsed with 5µCi (methyl-^{3}H) thymidine (specific activity 5Ci/mmole, Amersham, Buckinghamshire, England). Cultures were harvested on glass fiber filters (Whatman, Clifton, NJ) with a multiple automated sample harvester (Skatron Titertek, Lierbyen, Norway) and 7 mls PPO (2,5-Diphenyloxazole) was added. Dried filters were transferred to counting vials and the proliferative response was determined by measuring the radioactivity on a Packard Liquid Scintillation Counter (Packard Instrument Co., Downers Grove, ILL).

2.5 Immunisation

The appropriate antigen was dissolved in saline and emulsified in an equal volume of CFA (Difco Laboratories, Detroit, MI) containing 4mg/ml heat killed Mycobacterium butyricum (Difco Laboratories, Detroit, MI). Rats were immunised subcutaneously with a volume of 100 µl of the antigen/adjuvant emulsion into each hind footpad. The total dose received per rat was 50 µg antigen plus 400 µg M. butyricum.

2.6 Passive transfer of EAE with MBP reactive splenocytes

Single cell suspensions were prepared from the spleens of donor rats sensitized with 50µg MBP emulsified in CFA 10-12 days previously. Cells were cultured at $2x10^6$/ml in RPMI containing 5%FCS, $5x10^{-5}$M 2ME, 2mM L-glutamine and antibiotics. Cells were

activated *in vitro* with 2µg/ml Concanavalin-A (Con A) for 3 days at 37°C in an atmosphere of 10% CO_2, 7% O_2 and the balance N_2. Cells were harvested, washed in serum free medium and transferred via the lateral tail vein. Transfer populations contained $5x10^7$ viable cells (method of Panitch and McFarlin, 1977).

2.7 Generation of antigen-specific T cell lines

Activated MBP-, OA- and PPD-specific cell lines were derived from the lymph node cells of animals immunised with the appropriate antigen using the method of Ben-Nun et al., (1981) (Fig. 2.1). Draining lymph nodes (LNs) (popliteal and inguinal) were removed 10 days after immunization with protein antigens and single cell suspensions were prepared by gently pressing the LNs through a 400-mesh wire screen into RPMI (Flow, McLean, VA). Cells were washed once and resuspended at a concentration of $2x10^6$/ml in Eagle's medium (Grand Island Biological Co., Grand Is., NY) containing 1% fresh autologous serum, $5x10^{-5}$M 2-ME, 2mM L-glutamine, 1mM sodium pyruvate, antibiotics and 50 µg/ml of the appropriate antigen and incubated at 37°C in an atmosphere of 8% CO_2, 7% O_2 and the balance N_2. Following activation, cells were depleted of dead lymphocytes by density separation using Isopaque-ficoll as previously described by Davidson and Parish (1975). The cells collecting at the interface were found to be highly enriched for lymphoblasts. The cell lines were maintained in medium with a 15 % content of interleukin-2 (IL-2) containing medium (see below) and 10% FCS in the absence of specific antigen or accessory cells. The lines were alternately (every 3-4 days) stimulated with specific antigen (20µg/ml MBP, 50µg/ml OA, 50µg/ml PPD) in the presence of syngeneic irradiated (2000rads) normal thymus cells ($5x10^6$/ml) as accessory cells.

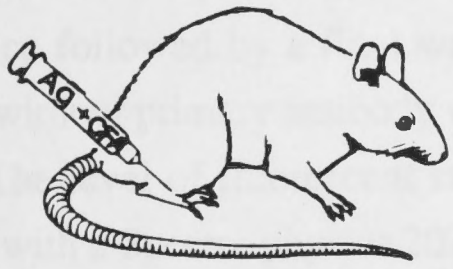
2.8 Interleukin-2 (IL-2) preparation

IL-2 containing culture supernatants were prepared based on the method of Rosenberg et al. (1978) and were used as a T cell growth factor for the maintenance of the cell lines. Single cell suspensions were prepared by gently pressing rat spleens through a 400-mesh wire screen into RPMI. Cells were washed once and resuspended at a concentration of 10^7/ml in RPMI containing 5%FCS, $5x10^{-5}$M 2ME, 2mM L-glutamine, antibiotics and 5µg/ml ConA and incubated for 4-6h at 37°C in an atmosphere of 8% CO_2, 7% O_2 and the balance N_2. Cells were washed twice by centrifugation (300g, 5mins, 4°C) to remove lectin and serum and resuspended at $5x10^6$/ml in serum free medium containing 100µg/ml bovine serum albumin (BSA). Cells were incubated for 40h at 37°C, then IL-2 enriched medium was harvested by centrifugation and sterile filtered.

PREPARATION OF SPECIFIC CELL LINES

Immunisation

ANTIGEN+CFA
INOCULATION



(10–12days)

REMOVAL OF
PERIPHERAL LYMPH NODES

Passive transfer

Autoimmune
Lymph Node
Cells

Alternate stimulation
with antigen and IL-2

Antigen Specific
T-cell line

Label with
H33342

ACTIVATED T-CELL
INOCULATION

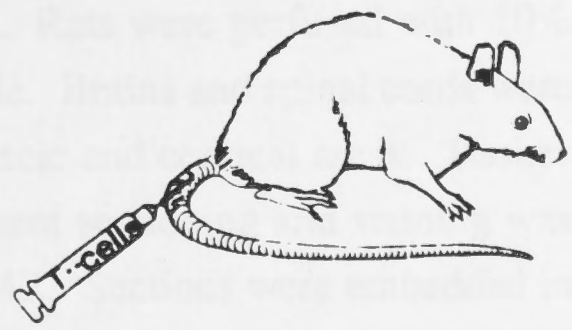


Fig. 2.1: Activated MBP-, OA- and PPD-specific cell lines were derived from the lymph node cells of animals immunised with appropriate antigen. Draining lymph nodes (popliteal and inguinal) were removed 10-12 days after immunization and single cell suspensions were prepared. The cell lines were maintained in medium with interleukin-2 and were alternately stimulated with specific antigen in the presence of accessory cells. Subsequently the lymph node derived cells lines were labelled with H33342 and injected via the tail vein into naive syngeneic recipients.

2.9 Immunofluorescence and flow cytometry

The surface phenotype of the MBP-specific T cell line was assessed by immunofluorescent flow cytometry using anti-CD4 (clone W3/25) and anti-CD8 (clone OX8, both kindly provided by Dr. Donald Mason, Oxford) monoclonal antibodies (mAb). MBP-specific cells (10^6) were resuspended in 200µl RPMI containing 5% FCS, 0.02% NaN_3, and the appropriate mAb dilution (1:50). Following incubation on ice for 60 mins the cells were underlayed with FCS, pelleted by centrifugation and incubated with 200 µl of 1:30 dilution of fluoresceinated sheep $F(ab')_2$ anti-mouse IgG (Silenus, Dandenong, Vic, Australia) for 30 mins on ice followed by a final wash through a layer of FCS. Secondary fluoresceinated antibody without primary antibody was used as a control for background staining.

The level of fluorescent staining was analysed on a Becton Dickinson FACS 440 equipped with a Spectra physics 2025 laser. Fluorescein was excited at 488nm, triggering on forward scatter through a number 1 neutral density filter and fluorescence emission detected through a 560 dichroic filter and 535/15 nm bandpass filter. Uncorrelated data was collected using log amplification with a base of 1 and multiplier of 0.5.

2.10 Clinical assessment of EAE

The animals were examined daily for clinical signs of EAE and scored on an arbitrary scale of severity ranging from 0 to 5 where: 0 = asymptomatic; 1 = flaccid distal half of tail; 2 = entire tail flaccid; 3 = ataxia, difficulty with righting reflex; 4 = hindlimb weakness; 5 = hindlimb paralysis. Animals suffering from hindlimb paralysis were given supportive care in that they were watered by hand, turned twice daily and dry bedding was maintained.

2.11 Histologic examination

Rats were selected for histological examination at various times post-immunisation with MBP in CFA or injection of effector cells. Rats were anaesthetized, the chest cavity was opened and a small incision was made in the right auricle. Rats were perfused with 10% neutral buffered formalin under pressure via the left ventricle. Brains and spinal cords were removed and the spinal cord was divided into lumbar, thoracic and cervical areas. Tissues were fixed in 10% neutral buffered formalin. The subsequent sectioning and staining was performed by Ms Wendy Hughes, Histology Section, JCSMR. Sections were embedded in paraffin, sectioned longitudinally and stained with either hematoxylin and eosin (Culling, 1974) to demonstrate cellular infiltrates in lesions, MSB Fibrin (method of Lendrum et al., 1962 as shown in Culling, 1974) to ascertain the degree of fibrin and collagen deposition, a mercuric chloride staining method (Naoumenko and Feigin's modification of Cajal's method as shown by Ralis et al., 1973) to demonstrate astrocytic hypertrophy throughout the CNS or chromoxane cyanine R (method of Pearse, 1957 as amended by Clark, 1981) which is a myelin sheath stain to observe demyelination (see below for the methods). A minimum of 10

sections from each area and at different levels was prepared from each animal for each staining method.

2.11.1 Hematoxylin and eosin stain

Sections of 4μms were:

1) deparaffinised and brought to water through xylene and graded alcohols;
2) stained with Gill's Hematoxylin (3mins);
3) washed in water;
4) treated with Scott's tap water substitute (0.2% sodium bicarbonate and 1.0% magnesium sulphate in distilled water) for 1 min;
5) washed in water;
6) stained in alcoholic eosin for 2 mins;
7) differentiated in 2 changes of 90% alcohol;
8) dehydrated and mounted.

Using a conventional microscope (Nikon Optiphot), the number and distribution of inflammatory lesions were assessed in five fields per section.

Stained sections were photographed under a conventional microscope (Nikon Optiphot) (Fig. 2.2a) and a laser scanning confocal microscope (Biorad MRC 500) (Fig. 2.2b).

2.11.2 MSB Fibrin stain

Sections of 4μms were:

1) placed in Zenker's solution overnight;
2) washed in running water for 1-2 hrs;
3) treated with a alcoholic iodine and sodium thiosulphate sequence to remove the mercury pigment;
4) washed in water;
5) stained with celestin blue (5mins);
6) rinsed in water;
7) stained with Gill's Hematoxylin (3mins);
8) washed in water;
9) treated with Scott's tap water substitute for 1 min;
10) washed in water;
11) rinsed in 95% alcohol;
12) stained with Martius yellow solution (2mins);
13) rinsed in distilled water;
14) stained in 1% brilliant crystal scarlet in 2.5% acetic acid for 10 mins;
15) rinsed in distilled water;

a)

b)

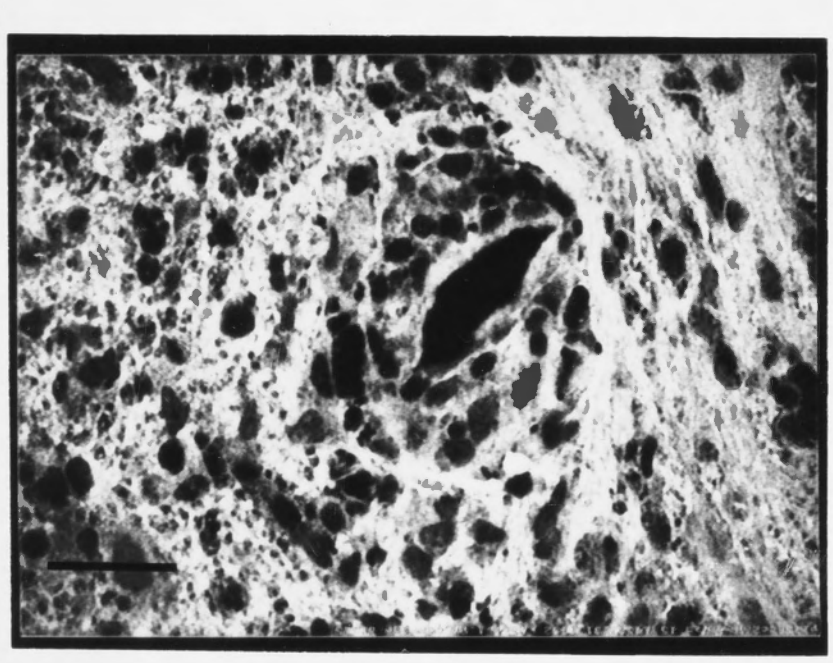


Fig. 2.2: Brain and spinal cord sections were stained using hematoxylin and eosin to demonstrate cellular infiltrates in lesions throughout the CNS. Stained sections were photographed under a conventional microscope (a) (x225, scale bar corresponds to 50μms) and a laser scanning confocal microscope (b) (x600, scale bar corresponds to 25μms).

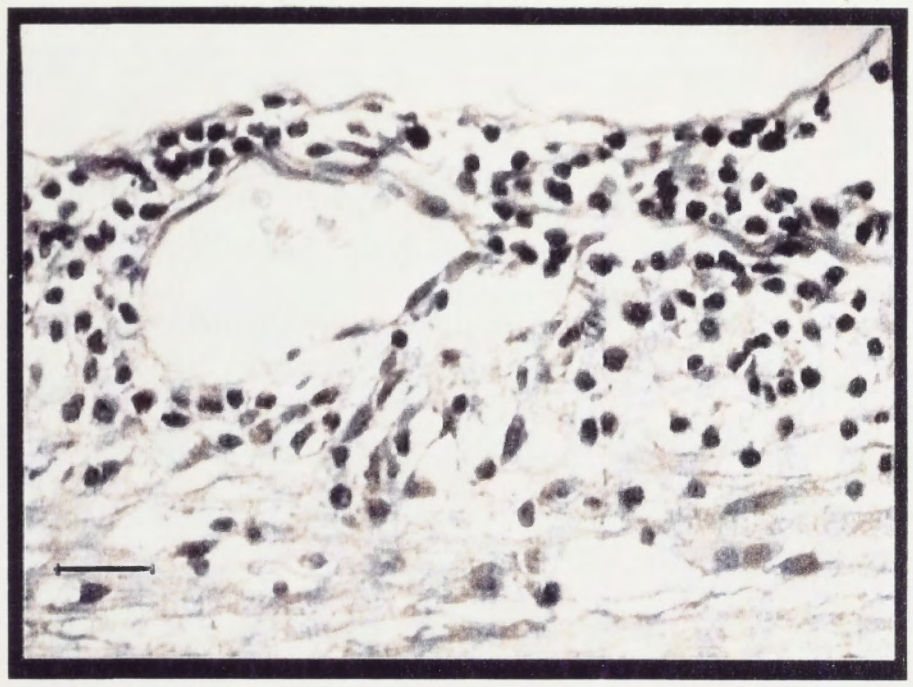
16) treated with 1% phosphotungstic for 5 mins;
17) rinsed in water;
18) stained in 0.5% aniline blue in 1% acetic acid 10mins;
19) rinsed in 1% acetic acid;
20) dehydrated and mounted.

Using this method the nuclei were stained blue black, fibrin was red, red cells were yellow and collagen was stained blue. Differences in fibrin deposition was determined using a conventional microscope (Nikon Optiphot). Stained sections were photographed under a conventional microscope (Nikon Optiphot) using the black and white video (Fig. 2.3a) or colour video (Fig. 2.3b) and a laser scanning confocal microscope (Biorad MRC 500) (Fig. 2.3c).

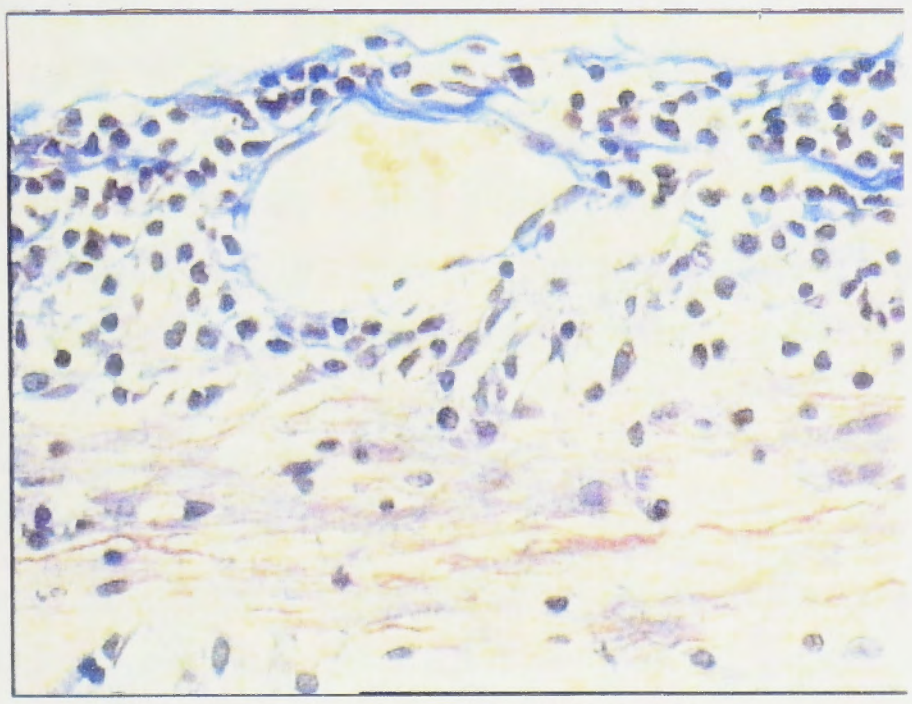
In addition, fibrin deposition was assessed by transmission electron microscopy in one animal from each group at each time point. Approximately four segments throughout the lumbar spinal cord were selected from each parraffin wax embedded specimen. Subsequently, the segments were prepared for electron microscopy by Ms Sue Bell (Histology Section, JCSMR) using the following procedure:

1) the wax was removed from the tissue using 2 changes of xylene for a total of 30 mins;
2) the tissue was rehydrated 3 times using a graded series of ethanols (absolute alcohol, 95%, 90%, 70%, 50%, 30%) for 10-15 mins each;
3) rinsed in distilled water for 1-2 mins;
4) immersed in 0.1M cacodylate buffer pH 7.4 for 1 hour;
5) and 2% osmium tetroxide solution for 1.5 hours;
6) washed 2 times with 0.1M cacodylate buffer (5 mins);
7) rinsed in distilled water;
8) stained with 2% uranyl acetate for 1 hour;
9) washed 2 times in distilled water (5 mins);
10) dehydrated 3 times in a graded series of ethanols (30%, 50%, 70%, 90%, 95%, absolute alcohol) for 10-15 mins each;
11) immersed in 1.1 mixture Spurr's resin/absolute alcohol for 2 hours;
12) and absolute Spurr's resin for 2 hours;
13) embedded in Spurr's resin and polymerised for 8 hours at 80^{o}C;
14) cut on a Reichert-Jung Ultracut ultramicrotome;
15) stained with uranyl acetate and Reynold's lead citrate;
16) examined and photographed on a Philips 301 Transmision Electron Microscope.

a)



b)



c)

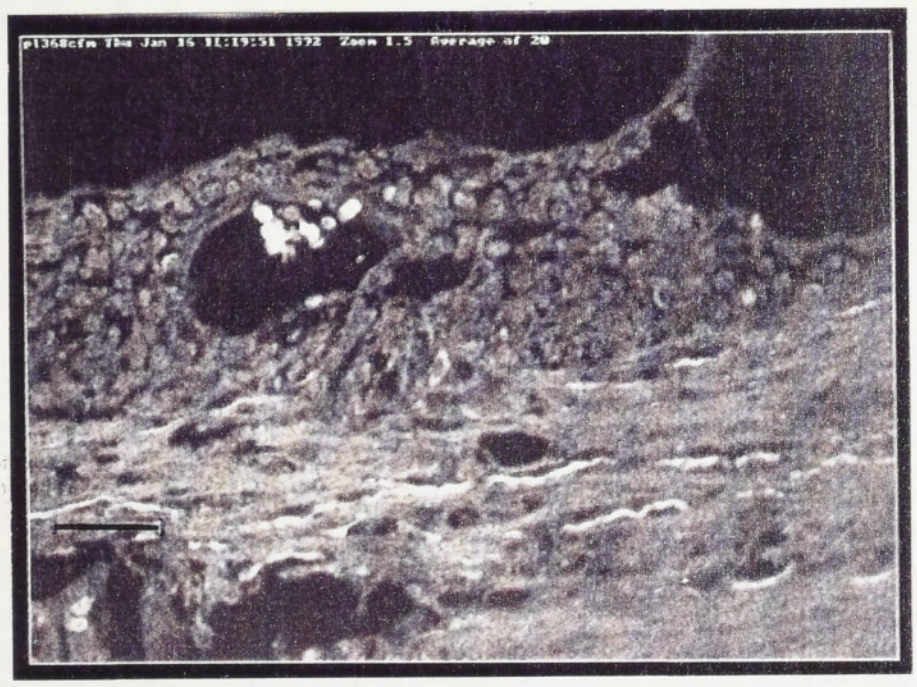


Fig. 2.3: Brain and spinal cord sections were stained using the MSB Fibrin technique to detect the degree of fibrin and collagen deposition. Using this method the nuclei were stained blue black, fibrin was red, red cells were yellow and collagen was stained blue. Stained sections were photographed under a conventional microscope using the black and white video (a) (x450, scale bar corresponds to 25µms) and colour video (b) (x450, scale bar corresponds to 25µms) and a laser scanning confocal microscope (c) (x200 scale bar corresponds to 100 µms).

2.11.3 Astrocyte Stain

Sections of 20µms were:

1) taken down to water and placed in a solution of 0.6g ammonium bromide and 14mls formalin in 100mls distilled water for 3 days at room temperature (RT);
2) rinsed briefly in distilled water;
3) placed in 8mls 1% gold chloride in 40 mls distilled water and 6.4mls of 5% aqueous mercuric chloride was added immediately and mixed well. Sections were kept in the dark for 12 hours at room temperature;
4) after rinsing for 2-5 mins, sections were fixed in 5% sodium thiosulphate for 5mins and rinsed again in distilled water for 5 mins;
5) dehydrated and mounted in Histoclad.

It has been found using this method that fibrous astrocytes of the white matter stain better than the grey matter and pathological astrocytes stain intensively while resting astrocytes are inconspicuous (Ralis et al., 1973). The astrocytes were stained dark purple with the background a pale pink colour. Using a conventional microscope (Nikon Optiphot), the changes in astrocyte staining patterns were assessed in five fields per section. Stained sections were photographed under a conventional microscope (Nikon Optiphot) (Fig. 2.4).

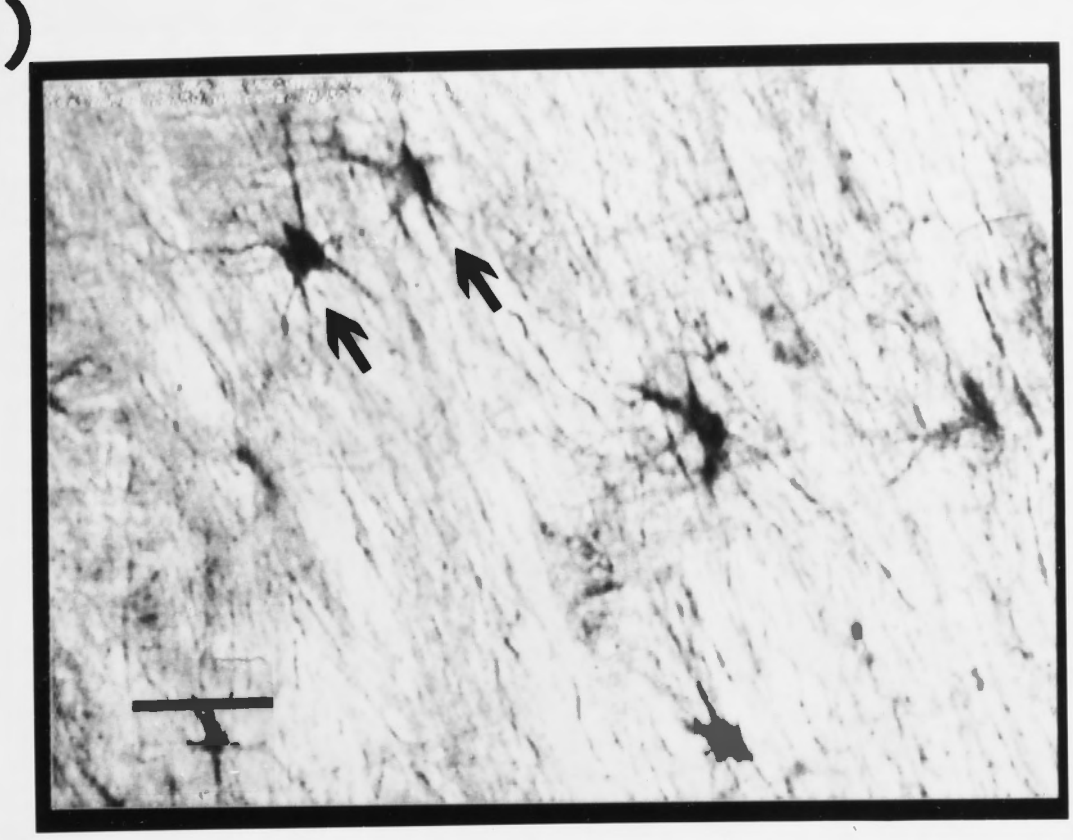
2.11.4 Chromoxane Cyanine R stain

Sections of 4µms were:

1) deparaffinised and brought to water through xylene and graded alcohols;
2) stained with chromoxane cyanine R solution (0.2% chromoxane cyanine R in 100mls 0.5% aqueous sulphuric acid, boiled for 5 mins) for 10 mins;
3) washed in water;
4) differentiated for 1 min in 1% aqueous ammonium hydroxide (NH_4OH);
5) washed in water;
6) counterstained 15 mins in 1% aqueous neutral red;
7) rinsed quickly in distilled water;
8) dipped 1 sec in copper sulphate-chrome alum solution (0.5gm $CuSO_4.5H_2O$, 0.5gm $CrK(SO_4)_3.12H_2O$, 3.0mls 10% acetic acid and 250mls distilled water);
9) rinsed quickly in distilled water;
10) dehydrated quickly and clear in xylene;
11) mounted.

Using this method the nuclei and nissl bodies were stained red and myelin was stained blue. Changes in myelin staining patterns were assessed using a conventional microscope (Nikon Optiphot) in five fields per section. Stained sections were photographed under a conventional microscope (Nikon Optiphot) using the colour video (Fig. 2.5a) and black and white video (Fig. 2.5b,c).

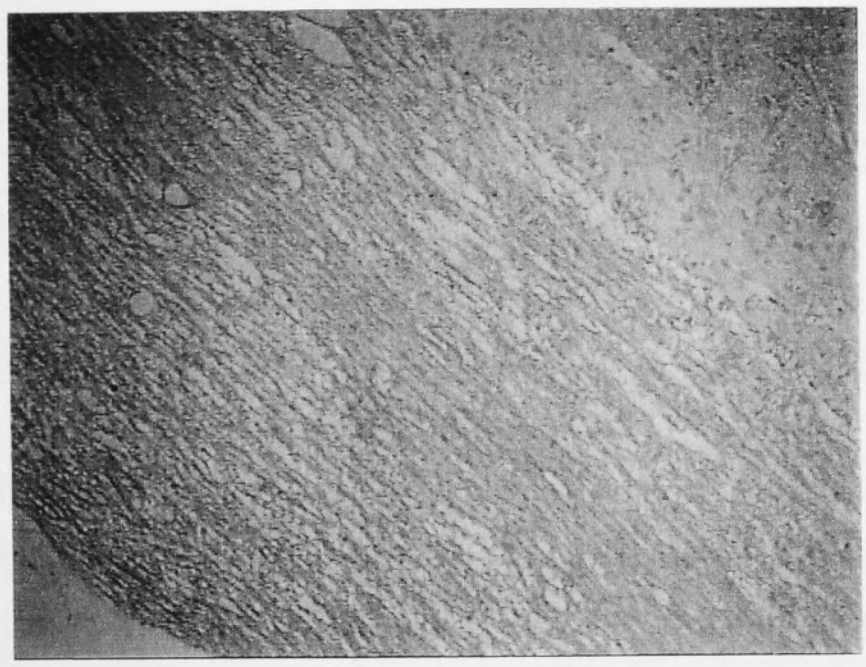
a)



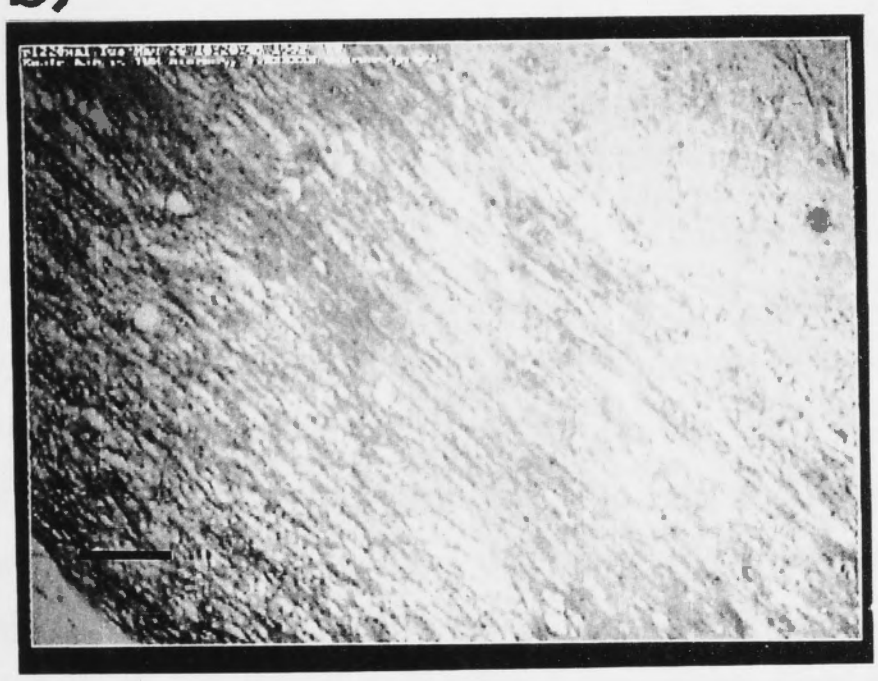
b)

Fig. 2.4: Brain and spinal cord sections were stained with a mercuric chloride technique to detect proliferating astrocytes. The astrocytes were stained dark purple with the background a pale pink colour. Stained sections were photographed under a conventional microscope (a) (x225, scale bar corresponds to 50μms) and (b) (x450, scale bar corresponds to 25μms).

a)



b)



c)

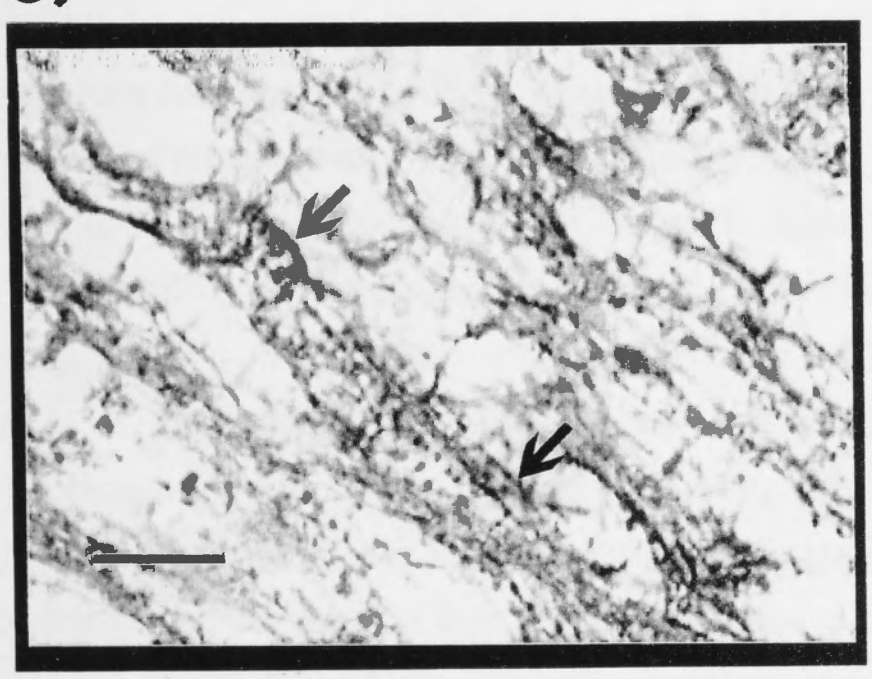


Fig. 2.5: Brain and spinal cord sections were stained using the chromoxane cyanine R staining technique to detect the degree of demyelination. Using this method the nuclei and nissl bodies were stained red and myelin was stained blue. Stained sections were photographed under a conventional microscope using the colour video (a) (x90) and black and white video (b) (x90, scale bar corresponds to 100μms) and (c) (x1350, scale bar corresponds to 10 μms, large arrows point to one myelinated axon).

2.12 Analysis of in vivo lymphocyte migration

The migration of lymphocytes was monitored *in vivo* by labelling cells with the DNA binding fluorescent dye Hoechst 33342 (H33342, Sigma, St. Louis, USA) as previously described by Brenan and Parish (1984). H33342 is an intensely fluorescent dye taken up by all cells, which remains intracellular, being diluted only by division (Brenan and Parish, 1984; Brenan et al., 1985; Weston and Parish, 1990).

In a pilot study, using a standard transfer population of 30 x 10^6 EAE cell lines, H33342 labelled cells were found to be as equally encephalitogenic as unlabelled cells. This was evidenced by identical disease critera of day of onset, severity and duration of symptoms (Willenborg, unpublished data).

Antigen-activated T cell lines were isolated 4 days after antigen stimulation *in vitro*. Non-activated cell populations were antigen-specific cell lines taken after 3 days culture in IL-2 containing medium. Lymphocytes were resuspended at $5x10^7$ cells/ml in RPMI containing 1%FCS to which was added 12µg/ml of H33342. The cells were incubated for 15 mins in a 37°C waterbath, being shaken after 10 mins. Labelling was stopped by adding cold medium, cells were washed twice by centrifugation (300g, 5 mins, 4°C) and resuspended in serum-free medium. Prior to injection the lymphocyte cell suspensions were examined for fluorescent intensity and uniformity of labelling. H33342 was found to have an intense fluorescence evenly distributed throughout the entire cell population. The cells were counted on a fluorescent microscope using a haemocytometer to ensure that the number of labelled cells to be injected was accurate and also checked for viability by their ability to exclude the dye trypan blue.

The H33342 labelled cells (10^8 viable cells) were then injected into a lateral tail vein of either irradiated or non-irradiated syngeneic rats. At specified time intervals the animals were observed for clinical signs of EAE, then anaesthetised with ether and 2.5 mls of blood collected by cardiac puncture and peripheral blood lymphocytes isolated by centrifuging the blood on Isopaque-ficoll. Subsequently, animals were killed and the lungs, spleen, mesenteric lymph nodes and spinal cord removed. The spinal cord was divided into three 20mm sections relating to the lower (lumbar 1-6, sacral 1), middle (thoracic 3-9) and the upper (cervical 1-7) regions (Fig. 2.6). Sections were weighed and single cell suspensions were then prepared by gently pressing tissue through a 400-mesh screen into 2 mls of medium. The content of H33342-labelled cells was quantified in the cell suspensions by fluorescence microscopy using an Olympus fluorescence microscope (BH series) and a HBO (100W) mercury vapour lamp for epi-illumination and appropriate barrier and filter combinations for H33342 (364nm excitation and >435nm emission). Labelling cells with H33342 resulted in an intensely fluorescent nuclear staining pattern which allowed cells to be easily observed against background autofluorescence at low magnification on a fluorescence

SPINAL CORD SECTIONS

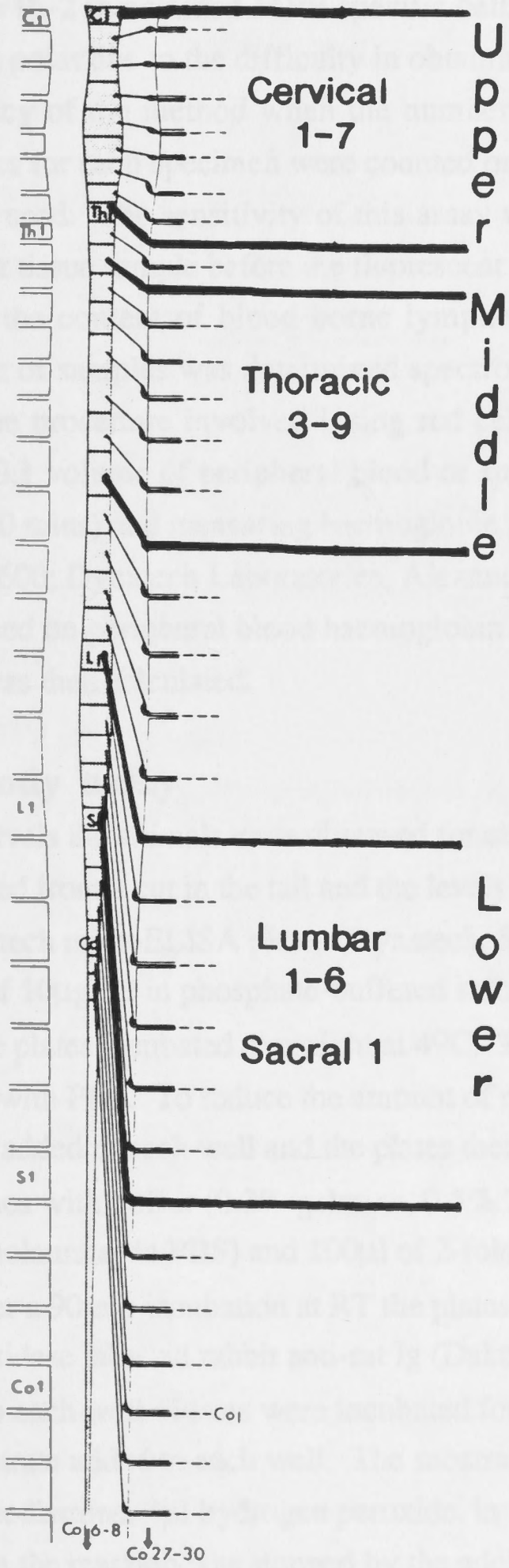


Fig. 2.6: Schematic diagram of the adult rat spinal cord showing the different regions of the spinal cord. In this study the spinal cord was divided into three sections relating to the lower (lumbar 1-6, sacral 1), middle (thoracic 3-9) and the upper (cervical 1-7) regions (Adapted from Waibl, 1973).

microscope. Positively stained cells could be accurately counted up to 4 days post inoculation.

The total number of labelled cells present in the organs or per 100mg spinal cord was then calculated. Determinations represent the mean of five animals for each group at each time point except in the case of the IL-2 maintained MBP-specific cells where data from only 3 animals was obtained per time point due to the difficulty in obtaining large numbers of these cells. To increase the accuracy of the method when the number of fluorescent cells in a sample was very low, 5 samples for each specimen were counted on the haemocytometer and the mean of these results was used. The sensitivity of this assay was such that at least 100 labelled cells was necessary per tissue sample before the fluorescent cells could be detected.

In order to estimate the content of blood borne lymphocytes in spinal cord cell suspensions, the blood content of samples was determined spectrophotometrically based on haemoglobin absorbance. The procedure involved lysing red cells by the addition of 1.9 volumes of distilled water to 0.1 volume of peripheral blood or spinal cord cell suspension, spinning out debris (12000g, 10 mins) and measuring haemoglobin absorbance at 540nm on a microplate reader (Model MR600; Dynatech Laboratories, Alexandria, VA) with a reference wavelength set at 630nm. Based on peripheral blood haemoglobin absorbance the content of blood in spinal cord samples was then calculated.

2.13 Anti-MBP antibody assay

At specified time intervals the animals were observed for clinical signs of EAE, anaesthetised with ether and bled from a cut in the tail and the levels of anti-MBP antibody in the serum was assessed. Dynatech microELISA plates (Dynatech, Switzerland) were coated with MBP at a concentration of 10µg/ml in phosphate-buffered saline (PBS). 200µl of MBP was added to each well and the plates incubated overnight at 4°C. The MBP was removed and the plates washed 3 times with PBS. To reduce the amount of non-specific binding, 200µl of 2% BSA in PBS was added to each well and the plates then incubated for 30min at RT. Plates were washed 3 times with buffer (0.2% gelatine, 0.5% Tween 20 (polyoxythlylene sorbitan monolaurate) in PBS) and 100µl of 2-fold dilutions (in buffer) of serum added to the wells. After a 90min incubation at RT the plates were washed 5 times with buffer and 100µl of peroxidase labelled rabbit anti-rat Ig (Dakopats, Copenhagen, diluted 1:1500 in buffer) was added to each well. Plates were incubated for 1 hr at RT, washed 5 times in buffer and 100µl substrate added to each well. The substrate was made fresh and consisted of 30mg O-phenylenediamine, 4µl hydrogen peroxide, in 10ml citrate-phosphate buffer pH 5.4. After 15-20min the reaction was stopped by the addition of 50µl 2M HCl to each well. Plates were read on a Dynatech microELISA plate reader at optical density $(OD)_{492nm}$. A panel of normal rat serum was run in each assay and the mean OD plus 3

standard deviations of this panel calculated. Any serum dilution higher than this value was considered positive. Titers are expressed as $\log_2$ serum dilution.

2.14 Measurement of TNF production

Detection of TNF in spinal cord and spleen tissue homogenates and biological fluids such as CSF and plasma was assessed. At specified time intervals the animals were observed for clinical signs of EAE, then anaesthetised with ether and 2mls of blood collected by cardiac puncture. Subsequently, animals were killed, CSF was removed with a 75mm micro-hematocrit tubes (Clay Adams) and the lumbar spinal cord removed. The blood was allowed to clot, serum removed and heat inactivated at 56°C for 30mins. Spinal cord sections were weighed and single cell suspensions were then prepared by gently pressing tissue through a 400-mesh screen into 2 mls of medium with 10% FCS added. CSF was diluted 1 in 25 with 10% FCS media.

Two different methods were performed to measure the production of TNF in the tissue homogenate and biological fluid samples of animals from different time points post inoculation.

2.14.1 Method 1

TNF levels were initially assessed using the MTT tetrazolium cytotoxicity assay (method of Mosmann, 1983 as shown in Espevik and Nissen-Meyer, 1986). Basically, target cells were seeded in 96 well flat-bottomed plates (Dynatech, Switzerland) at a concentration of $2x10^4$ cells/well (WEHI 164) in 100µl H16 medium containing 10% FCS, $5x10^{-5}$ 2ME and 2µg/ml actinomycin D. Different dilutions of TNF (r-Mu TNF, Genzyme, Boston, MA) in H16 were added to the target cells. After 20h of incubation at 37°C in CO_2, 10 µl MTT at a concentration of 5mg/ml in PBS was added and further incubated for 4-6 h at 37°C in CO_2. 100µl of isopropanol with 0.01 M HCL was added to all wells and incubated overnight. The plates were read on a Dynatech MR 600 microplate reader, using a test wavelength of 600nm and a reference wavelength of 630nm. The percentage of dead cells was determined as the OD in wells with TNF divided by the OD in control wells multiplied by 100.

2.14.2 Method 2

TNF-α levels in the tissue homogenates and biological fluids from two different experiments were determined using the ELISA method based on the method of Sheehan (1989) (Wheeler et al., 1991) by Sue Fordham (Division of Cell Biology, JCSMR) and Elizabeth Rockett (Dept. of Biochemistry and Molecular Biology, ANU). Briefly, 96 well flat-bottomed plates (Immulon II, Dynatech Labs, Alexandria, VA) were coated by overnight incubation at 4°C with 0.2µg per well of TNF319:12, a TNF-specific mAb, which had been made up in carbonate buffer (pH 9.6). The plates were washed 6 times with PBS containing

0.05% Tween 20, and again between each subsequent treatment. They were then incubated overnight at 4°C with 100µl of a 1 in 5 dilution of the test samples per well in RPMI 1640 supplemented with 10% FCS, 10mM penicillin and streptomycin, and 10mM HEPES, (R10). A standard curve of recombinant murine TNF-α (Genzyme, Boston, MA) starting at 25ng/ml and followed by 8 doubling dilutions, was used. Rabbit anti-murine TNF-α (100µl, Genzyme) in R10 at 1 in 750 dilution was added to each well for 2 hours at 25°C. The same volume of goat anti-rabbit alkaline phosphatase (Silenus, Victoria, Australia) at a dilution of 1 in 1000 in R10 was then added for 2 hours at 25°C. Colour development was achieved using 100µl per well of phosphatase substrate tablets (Sigma, St. Louis, MO) dissolved in substrate buffer to a concentration of 1mg/ml. The plates were then read with a Titertek Multiskan MC ELISA reader (Flow Labs, McLean, VA) at a wavelength of 450nm with a reference of 620nm, and the TNF-α in the test samples calculated from the standard curve.

2.15 Determination of blood glucose levels

Glucose levels in the blood were determined by the method of Simeonovic et al. (1990). At specified time intervals the animals were observed for clinical signs of EAE, then anaesthetised with ether and a sample of blood (10µl) was taken from the tail vein and diluted in 40µl of 0.66M perchloric acid. After centrifugation at 15,000 rpm for 2.5 mins (Beckman microcentrifuge), the plasma glucose levels (mmol/l) was measured in a Beckman Glucose Analyser 2 (Beckman Instruments, Inc., Fullerton, CA). The instrument was calibrated using the manufacturer's standard and each sample was measured in duplicate.

2.16 Determination of glucocorticosteroid levels

Middle aged (12-14mths) and young (2-3mths) male animals were caged in pairs. Between the hours of 9:00 and 10:00 a.m. both animals were anesthetised simultaneously and bled from a cut in the tail within 2 min. This procedure does not elevate blood steroid levels significantly within this time interval (Cook et al., 1973; MacPhee et al., 1989). Blood was allowed to clot, kept at 4°C for 24 hrs, and then centrifuged (15,000rpm, 10mins) on a Beckman microcentrifuge. The serum was removed and stored at -20°C until assayed. The level of corticosterone in the serum was determined by a radioimmunoassay (RIA) using the RSL (^{125}I) corticosterone assay system (ICN Biomedicals, Inc., Costa Mesa, CA).

2.17 Determination of prostaglandin E levels

2.17.1 The removal of spinal cord tissue

The techniques employed in the preparation of spinal cords of normal and sensitised rats for PGs extraction were based on the method of Bolton et al. (1984a). At specified time intervals the animals were observed for clinical signs of EAE, then killed with CO_2 and the lumbar spinal cord exposed and washed in situ with a chilled solution of PBS containing 5%

(w/v) sodium bicarbonate and 10μg/ml of the PG synthetase inhibitor, indomethacin (Sigma, St. Louis, MO). The drug was employed to minimise the extracellular synthesis of PGs which are released by physical disruption (Samuelsson et al., 1975; Vapaatalo and Parantainen, 1978) during the dissection procedure. Each spinal cord section was weighed and then transferred to glass universals containing 2 mls of chilled PBS/indomethacin solution. Samples were homogenised at 4°C for 5 mins, and the resulting homogenates were centrifuged (3000rpm, 15 mins at 4°C). Supernatants were aliquoted, gassed with nitrogen for 30 secs and stored at -70°C.

2.17.2 Extraction of PGs from supernatants

In glass universals, supernatant volumes of 0.5ml were diluted 1 in 2 with PBS, adjusted to pH3.0 with 1N HCl (tested on litmus paper) and extracted 3 times with diethyl ether. The ether was removed by evaporation using nitrogen gas and the dried extracts were stored under nitrogen at -70°C until assayed.

2.17.3 Radioimmunoassay (RIA) of extracted supernatants

Dried supernatant extracts were solubilised in 1 ml of 0.1M tricine-buffered saline containing 0.1% gelatine and 0.1% sodium azide. RIA was undertaken using a PGE_2 (^{125}I) assay system (Code RPA 530, Amersham, England).

2.18 Determination of reactive nitrogen intermediate levels

At specified time intervals the animals were observed for clinical signs of EAE, then anaesthetised with ether and 2mls of blood collected by cardiac puncture. Subsequently, animals were killed, CSF was removed with a 75mm micro-hematocrit tubes (Clay Adams) and the lumbar spinal cord removed. Blood was allowed to clot, serum removed and heat inactivated at 56°C for 30mins. Spinal cord sections were weighed and single cell suspensions were then prepared by gently pressing tissue through a 400-mesh screen into 2 mls of medium with 10% FCS added. CSF was diluted 1 in 25 with 10% FCS media.

The measurement of reactive nitrogen intermediates (RNI) on tissue homogenates and biological fluids was performed by Melissa Awburn (Division of Cell Biology, JCSMR). The assay was based on the reduction of nitrate by a Cd-Cu complex (Davidson and Woof, 1978; Rockett et al., 1991) and coupled to the Griess reagent for azochromaphoretic detection. Basically, 30μl volumes of plasma, cell suspensions or CSF were dispensed into 1.5ml plastic tubes (Elkay Products Inc., Shrewsbury, MA) in duplicate. Sodium nitrite (Sigma Chemical Co., St. Louis, MO) and sodium nitrate (Sigma) standards (concentration range: 1μM to 1mM) were set up separately in 30μl volumes of pooled normal mouse serum. Control tubes contained 30μl of normal mouse plasma or 30μl of phosphate buffered saline (pH7.2) (blank). Twenty microlitres of catalyst buffer was added to one set of samples and

the nitrite standard curve, while the other set and the nitrate standard curve received 20µl of catalyst. All the tubes were incubated at room temperature for 5 mins, after which the tubes containing the catalyst were centrifuged at 15,000rpm for 1 min (Beckman microcentrifuge) and the supernatants were transferred to clean tubes. Griess reagent (100µl) and 10% trichloroacetic acid (100µl) were added to all tubes and mixed thoroughly before centrifuging for 15 mins at 15,000rpm (Beckman microcentrifuge). Two 100µl samples of the supernatant from each tube were transferred to a 96-well flat-bottom plate (Nunc, Denmark) and the absorbance was read on a microplate reader (Dynatech MR 600, Dynatech Scientific, Inc., Cambridge, MA), using a test wavelength of 540nm and a reference wavelength of 630nm.

Plasma nitrite was calculated by reading the absorbance directly from the nitrite standard curve, whereas reading plasm nitrate from the nitrate standard curve first required the absorbance of the sample (without catalyst) to be subtracted from the absorbance of the sample (with catalyst). The results are expressed as micromolar concentrations of total RNI (the sum of nitrite and nitrate) concentrations.

2.19 Statistical Analysis

Statistical analysis was carried out using the Student t-test. The means of various treatments were considered to be significantly different when $P<0.05$.

CHAPTER 3: SELECTIVE LOCALISATION OF NEURO-SPECIFIC T LYMPHOCYTES IN THE CNS

3.1 Introduction

A critical feature in the pathogenesis of EAE is the entry of T lymphocytes into the parenchyma of the CNS. This is the case whether EAE is induced actively by immunisation with MBP emulsified in CFA or passively with activated MBP-specific CD4+ T cells (Smith and Waksman, 1969; Raine, 1976; Hickey et al., 1983; Fontana et al., 1984; Traugott et al., 1986; Zamvil and Steinman, 1990). The mechanism of this initial entry into the CNS is not known, and the concept that antigen recognition at the endothelial cell surface triggers migration would seem no longer tenable (Lassman et al., 1986 ; Hinrichs et al., 1987).

Wekerle et al. (1986) have proposed that the initial emigration of lymphocytes in the CNS is not immunologically specific. They hypothesise that "activated" T lymphocytes of any specificity migrate across the CNS endothelium into the parenchyma of the CNS and that this random migration acts as an immune surveillance mechanism. If cells entering the CNS encounter an antigen for which they have a specificity, they respond accordingly, otherwise they either die or move back out of the CNS. Willenborg and Parish (1988) expanded this hypothesis to suggest that "activation" translated to the expression of enzymes on the lymphocyte surface which assisted the cells in degrading the sub-endothelial basement membrane and the extracellular matrix, thus facilitating movement through interendothelial junctions and the subendothelial extracellular matrix into the parenchyma. Both direct and indirect evidence has been provided to support this hypothesis. Activated lymphocytes produce elevated levels of a heparan sulphate-specific endoglycosidase (Naparstek et al., 1984; Lider et al., 1989) which specifically degrades the heparan sulphate side chains of the proteoglycan scaffold of the extracellular matrix. Inhibitors of this enzyme also prevent EAE development (Naparstek et al., 1984; Lider et al., 1989; Willenborg and Parish, 1988). Non-activated cells which lack the elevated levels of heparan sulphate endoglycosidase (Naparstek et al., 1984; Lider et al., 1989) do not cause disease (Panitch and Ciccone, 1981; Ben-Nun et al., 1981; Holda et al., 1980; Peters and Hinrichs, 1982).

The study described in this chapter explores further the pathogenesis of EAE by quantifying the accumulation and localisation of Hoechst 33342 (H33342)-labelled lymphocytes of different specificities in different regions of the spinal cord. H33342 is a highly fluorescent dye which binds specifically to DNA (Brenan and Parish, 1984; Brenan et al., 1985; Weston and Parish, 1990) and individual fluorescent cells can be quantified by

fluorescence microscopy. The hypothesis that "activated" lymphocytes of any specificity enter the CNS but only those with neuro-antigen specificity persist and cause pathology was addressed in this study. The data presented in this chapter strongly support the hypothesis as T cell lines specific for MBP, purified protein derivative of tuberculin (PPD), or ovalbumin (OA) all enter the spinal cord, if first activated with their specific antigen, but only MBP-specific cell lines accumulate. OA- and PPD-specific cell lines were used because the antigen-specificities of these lines are non-specific to the CNS and cause no known illness or inflammation in syngeneic rats. Furthermore, IL-2 maintained (non-activated) MBP-specific cell lines fail to enter the CNS and cause disease. The host contribution to passively induced EAE has also been examined by assessing whether the entrance and accumulation of activated MBP-specific cells is altered by irradiation of the recipients.

3.2 Results

3.2.1 Characterisation of cell lines

A number of cell lines were established which were MBP-specific or specific for the non-neural antigens OA and PPD. A consistent feature of all cell lines was that they remained viable when cultured in IL-2 containing medium, although there was usually no increase in cell numbers, whereas it was usual to expect a 4-10 fold increase in cell numbers following activation with specific antigen. Immunofluorescent flow cytometry revealed that after two to three months of alternately culturing with specific antigen or IL-2 the cell lines were predominantly T cells with approximately 85-95% of the cells being CD4+ and CD8- (Fig. 3.1).

It was found using a blastogenesis assay that our PPD cell line responded marginally to MBP (at 25μg/ml of MBP, the PPD-specific cell line had a stimulation index of 2.3±0.21, compared with the MBP-specific cell line stimulation index of 10.25±1.17). Cross-reactivity between PPD and MBP has also been described previously by Vandenbark et al. (1975).

3.2.2 Ability of cell lines to induce EAE

To assess the ability of various cell lines to induce EAE, the antigen-activated MBP-specific cell line, the IL-2 maintained MBP-specific cell line and the antigen-activated OA-specific cell line were all labelled with H33342 and then transfered at 10^8 cells per rat into naive recipients. As can be seen in Fig. 3.2 the non-specific T cell line (OA) and the IL-2 maintained (non-activated) MBP cell line failed to induce disease whereas the activated

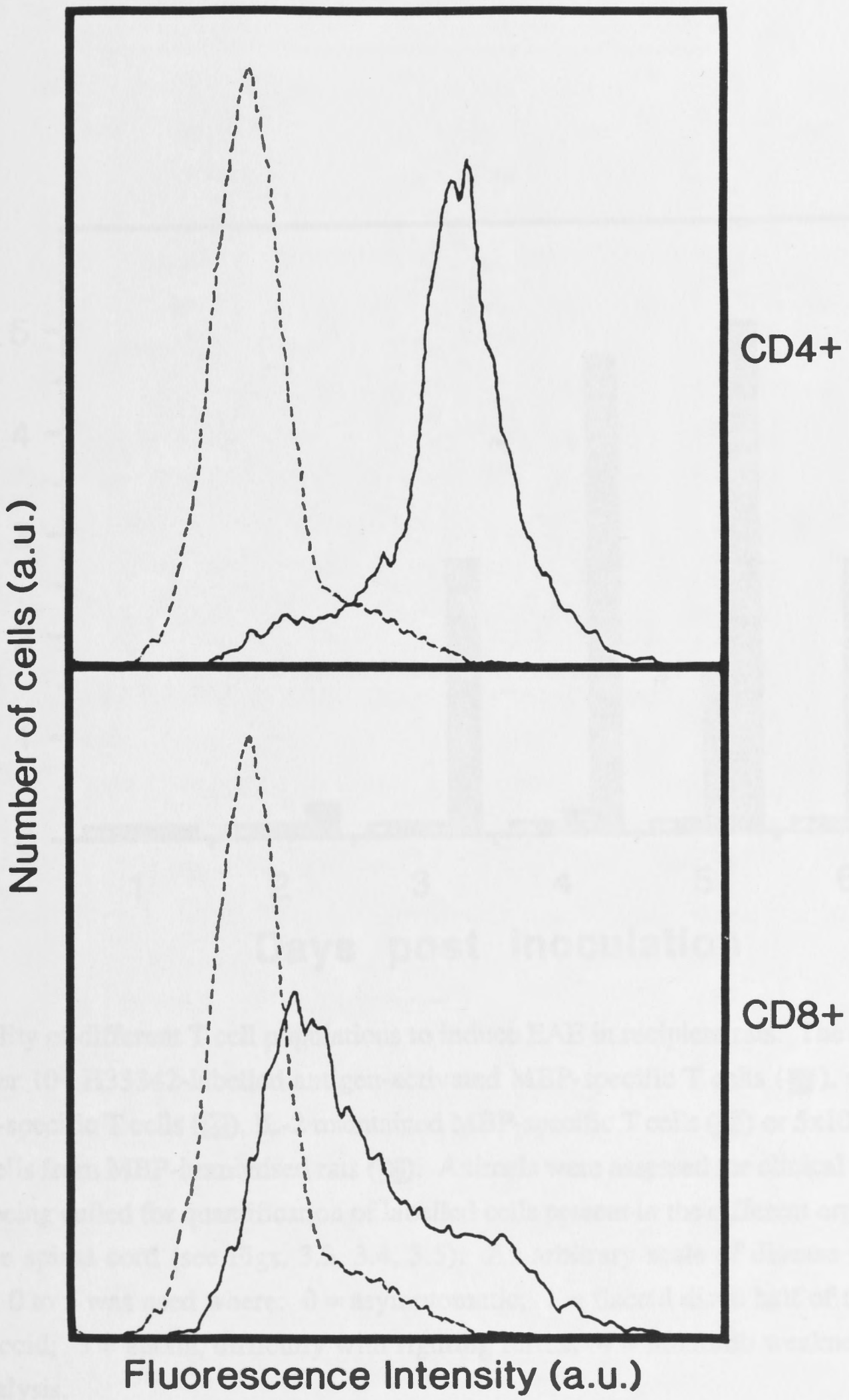


Fig. 3.1: Fluorescence profiles measured by flow cytometry of MBP-specific cell line labelled with (a) W3/25 (a marker for CD4+ T cells) and (b) OX8 (a marker for CD8+ T cells) monoclonal antibodies.

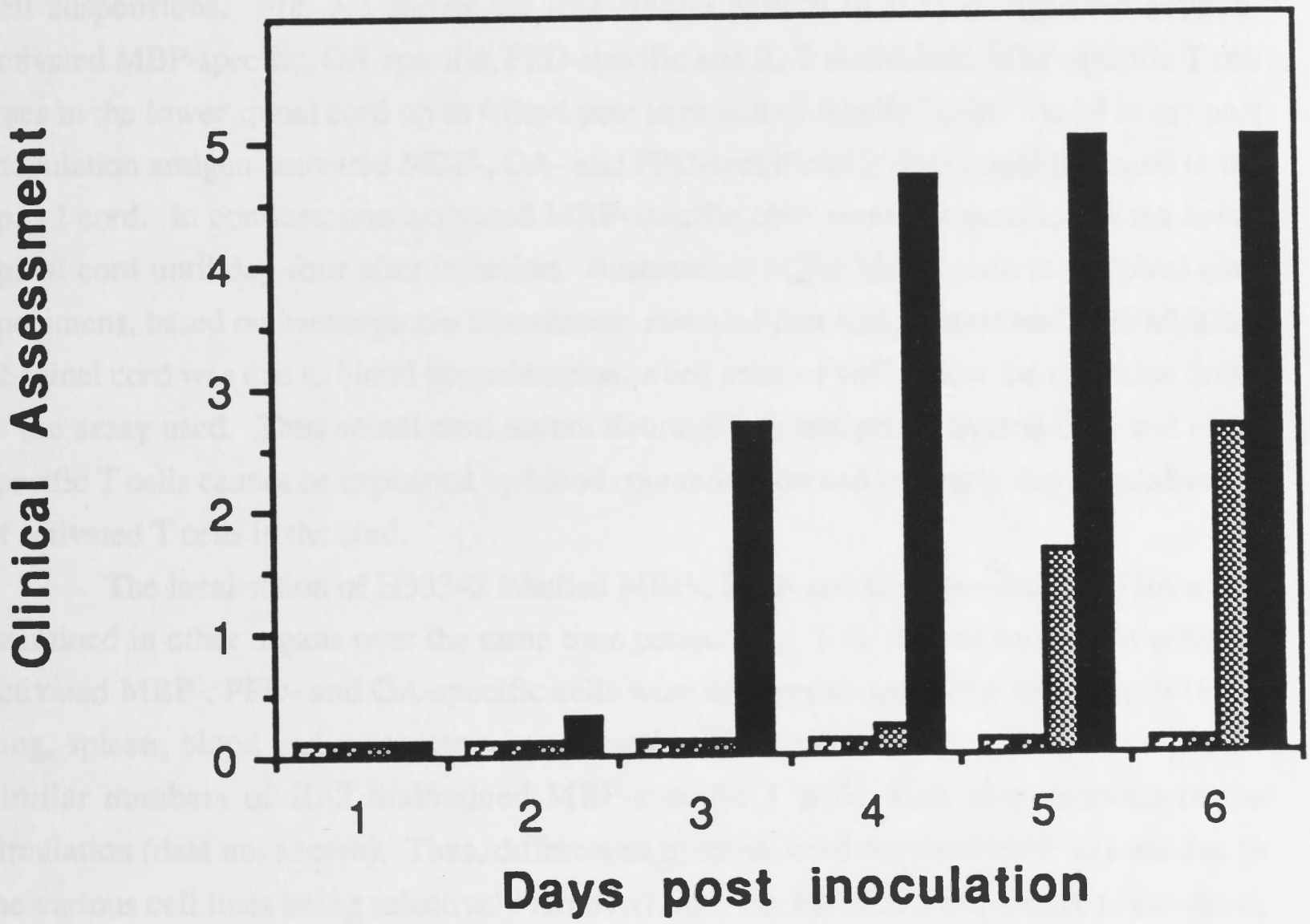


Fig. 3.2: Ability of different T cell populations to induce EAE in recipient rats. The animals received either 10^8 H33342-labelled antigen-activated MBP-specific T cells (■), antigen-activated OA-specific T cells (▨), IL-2 maintained MBP-specific T cells (▨) or $5x10^7$ ConA activated T cells from MBP-immunised rats (▩). Animals were assessed for clinical signs of EAE before being culled for quantification of labelled cells present in the different organs and regions of the spinal cord (see Figs. 3.3, 3.4, 3.5). An arbitrary scale of disease severity ranging from 0 to 5 was used where: 0 = asymptomatic; 1 = flaccid distal half of tail; 2 = entire tail flaccid; 3 = ataxia, difficulty with righting reflex; 4 = hindlimb weakness; 5 = hindlimb paralysis.

MBP cell line caused disease beginning as early as day 2 after cell transfer. The disease produced by 10^8 MBP line cells was much more severe than that caused by the standard dose of ConA activated EAE spleen cells as would be expected of a cell line. This large number of cells was chosen in order to increase the chances of detecting migration of small numbers of cells.

3.2.3 Tissue localisation of fluorescently labelled cells

The localisation of the H33342 labelled cell populations in different organs of recipient rats was assessed at various times post inoculation by fluorescence microscopy of cell suspensions. Fig. 3.3 shows the localisation pattern of H33342-labelled antigen-activated MBP-specific, OA-specific, PPD-specific and IL-2 maintained MBP-specific T cell lines in the lower spinal cord up to 4 days post injection of labelled cells. At 24 hours post inoculation antigen-activated MBP-, OA- and PPD-specific cell lines could be found in the spinal cord. In contrast, non-activated MBP-specific cells were not detected in the lower spinal cord until day four after injection. Assessment of the blood content of spinal cord specimens, based on haemoglobin absorbance, revealed that <30 fluorescent T cells/100mg of spinal cord was due to blood contamination, a cell number well below the detection limits of the assay used. Thus spinal cord accumulation of the antigen-activated OA- and PPD-specific T cells cannot be explained by blood contamination and is clearly due to localisation of activated T cells in the cord.

The localisation of H33342 labelled MBP-, PPD- and OA-specific T cell lines was examined in other organs over the same time period (Fig. 3.4). It was found that antigen-activated MBP-, PPD- and OA-specific cells were at approximately the same levels in the lung, spleen, blood and mesenteric lymph nodes of recipient animals at all time points. Similar numbers of IL-2 maintained MBP-specific T cells were also detected in the circulation (data not shown). Thus, differences in spinal cord accumulation was not due to the various cell lines being selectively removed from the blood and sequestered elsewhere, so that fewer cells were available in the circulation.

The MBP activated cell line showed a dramatic increase in spinal cord accumulation with time. At day 2, one day before the appearance of clinical signs (Fig. 3.2), there was a 30 fold increase in the number of labelled MBP-specific T cells in the lower spinal cord with further increases occurring at days 3 and 4 (Fig. 3.3). In fact, at 4 days post injection there was up to a 1000 fold greater number of MBP-specific T cells in the CNS than OA-specific T cells.

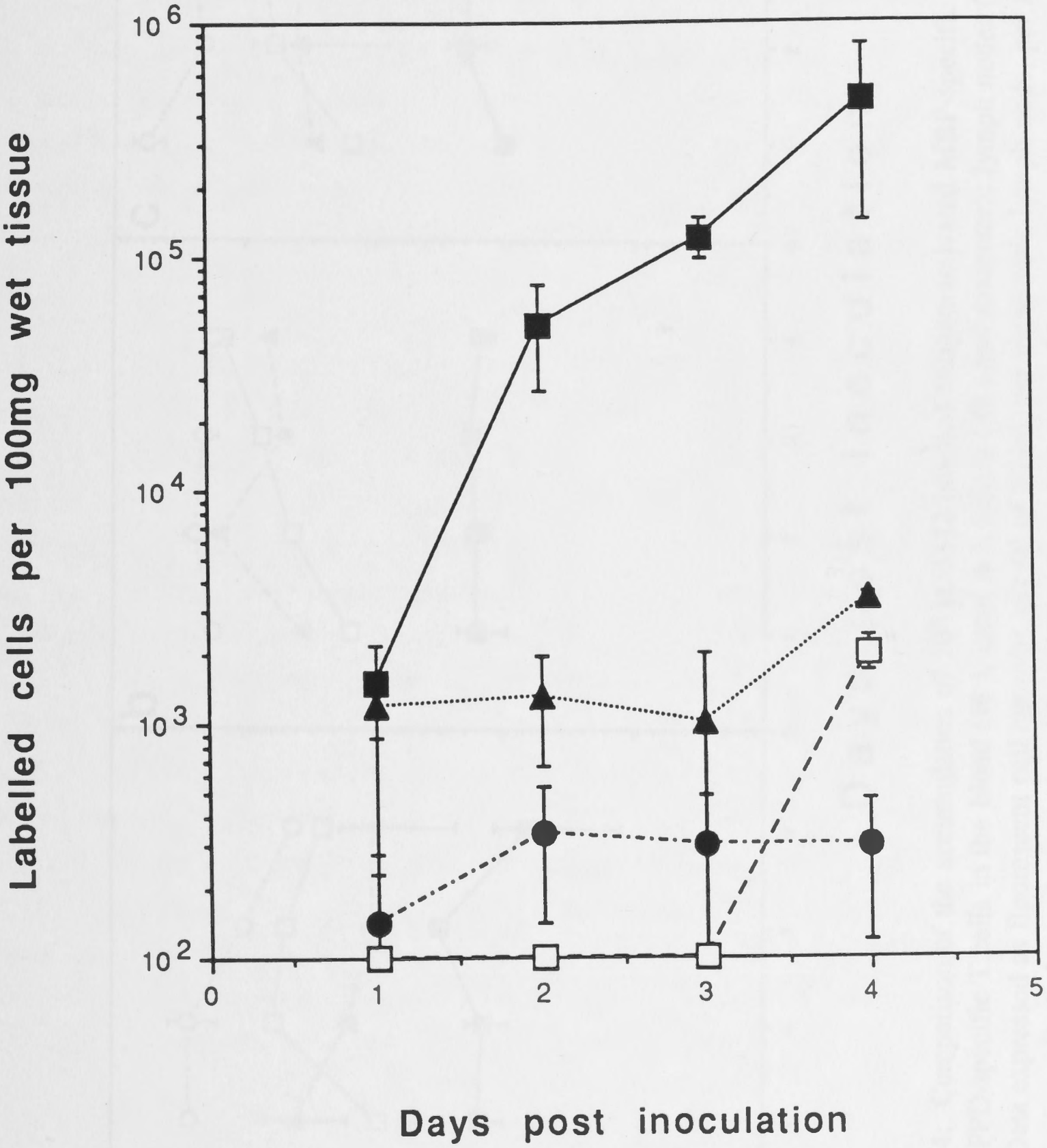


Fig. 3.3: The time dependent accumulation of 10^8 H33342 labelled antigen-activated MBP-specific (■), OA-specific (●), PPD-specific (▲) or IL-2 maintained MBP-specific (□) T cells in the lower spinal cord (lumbar segments 1-6, sacral segment 1) of recipient rats. Each data point represents the mean and standard error of 5 animals except with the IL-2 maintained MBP-specific cell population where each data point represents the mean of 3 animals.

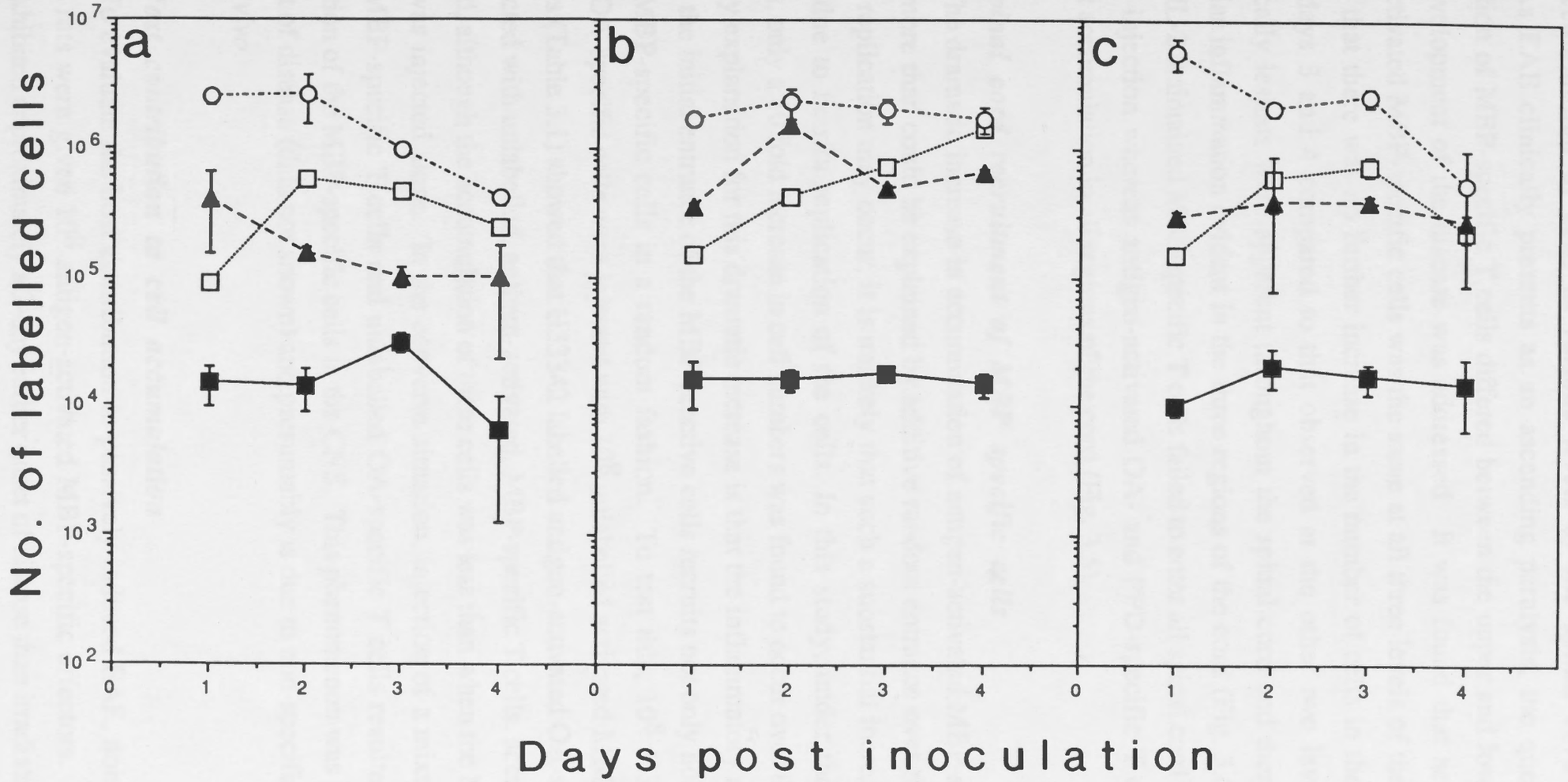


Fig. 3.4: Comparison of the accumulation of 10^8 H33342 labelled antigen-activated MBP-specific (a) OA-specific (b) or PPD-specific T cells in the blood (■), lung(▲), spleen (O) and mesenteric lymph nodes (□) of recipient rats. Data expressed as fluorescent cell numbers per ml of blood, per mesenteric lymph node, per left lung and per whole spleen. Each data point represents the mean and standard error of 5 animals.

3.2.4 Accumulation of cells at various levels of the spinal cord

As EAE clinically presents as an ascending paralysis, the question whether accumulation of MBP-specific T cells differed between the upper and lower spinal cord during development of the disease was addressed. It was found that accumulation of antigen-activated MBP-specific cells was the same at all three levels of the cord with the exception that there was no further increase in the number of cells in the cervical cord between days 3 and 4 compared to that observed at the other two levels (Fig. 3.5). Histologically lesions were apparent throughout the spinal cord and there was marked perivascular inflammation evident in the three regions of the cord (Fig. 3.6). It was also clear that IL-2 maintained MBP-specific T cells failed to enter all spinal cord regions up to 3 days post injection whereas antigen-activated OA- and PPD-specific T cells exhibited significant accumulation in all regions of the cord (Fig. 3.5).

3.2.5 Spinal cord recruitment of MBP specific cells

The dramatic increase in accumulation of antigen-activated MBP-specific cells is certainly more than could be explained by additive random entrance over time. Although some cell replication may occur, it is unlikely that such a substantial increase (1000 fold) could be due to *in situ* replication of the cells. In this study, under the best *in vitro* conditions, only a 10 fold increase in cell numbers was found to occur over three days. The most likely explanation for this dramatic increase is that the inflammatory response set up following the initial entrance of the MBP-reactive cells recruits not only non-specific cells but also MBP-specific cells in a random fashion. To test this, 10^8 H33342 labelled activated OA-specific cells were injected with 10^8 unlabelled activated MBP-specific cells. The results (Table 3.1) showed that H33342 labelled antigen-activated OA-specific T cells, when injected with unlabelled, antigen-activated, MBP-specific T cells, accumulated in the spinal cord, although the accumulation of these cells was less than when the MBP-specific T cell line was injected alone. In the converse situation, injection of a mixture of H33342 labelled MBP-specific T cells and unlabelled OA-specific T cells resulted in a reduced accumulation of the MBP-specific cells in the CNS. This phenomenon was parallelled by a later onset of disease (data not shown) and presumably is due to non-specific cell crowding effects *in vivo*.

3.2.6 Host contribution to cell accumulation

To evaluate the host contribution to passively induced EAE, non-irradiated and irradiated rats were given 10^8 antigen-activated MBP-specific effectors. Non-irradiated animals exhibited approximately a 2-day earlier onset of disease than irradiated animals (Fig.

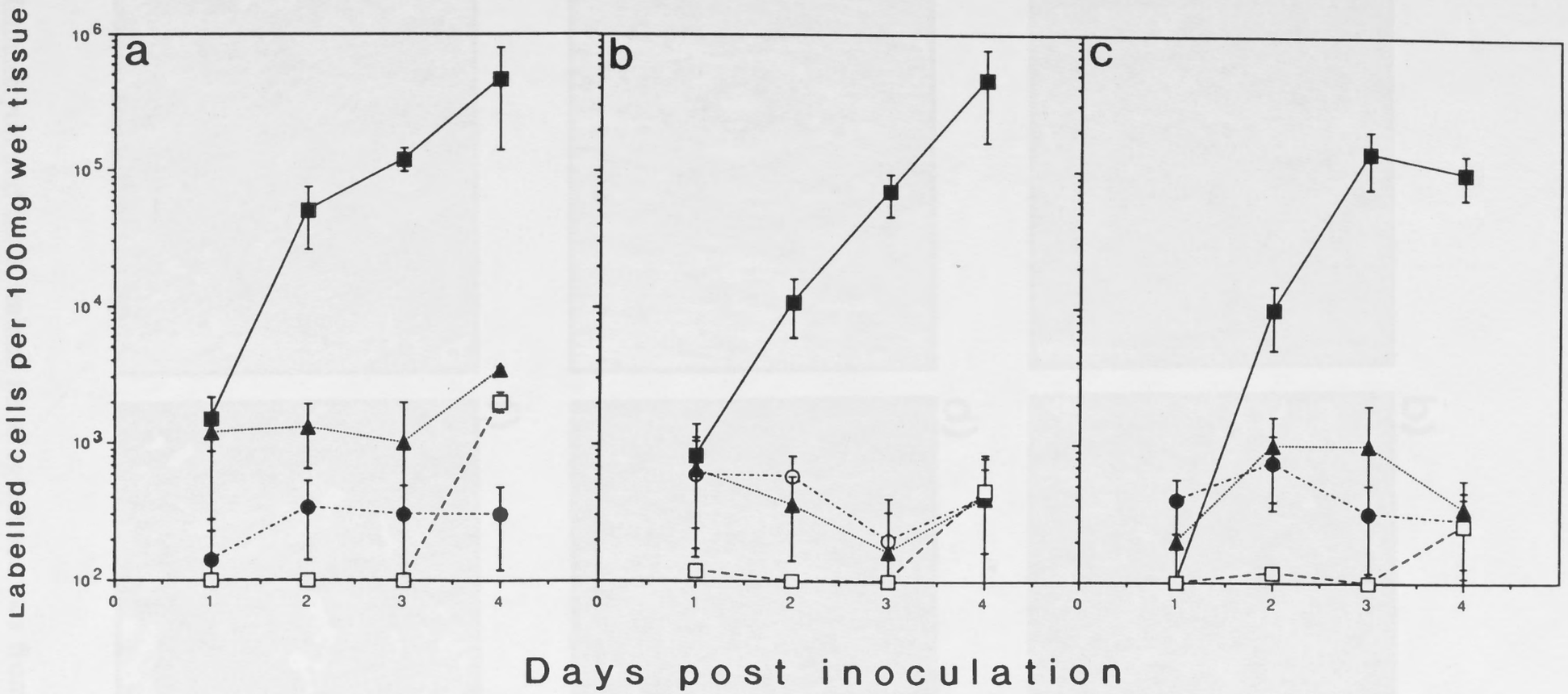


Fig. 3.5: The time dependent accumulation of 10^8 H33342 labelled antigen-activated MBP-specific (■), OA-specific (●), PPD-specific (▲) or IL-2 maintained MBP-specific (□) T cells in the spinal cord of recipient rats. Data presented for fluorescent cell accumulation in (a) lower spinal cord (lumbar segments 1-6, sacral segment 1); (b) middle spinal cord (thoracic segments 1-9); and (c) upper spinal cord (cervical segments 1-7). Each data point represents the mean and standard error of 5 animals except with the IL-2 maintained MBP-specific cell population where each data point represents the mean of 3 animals.

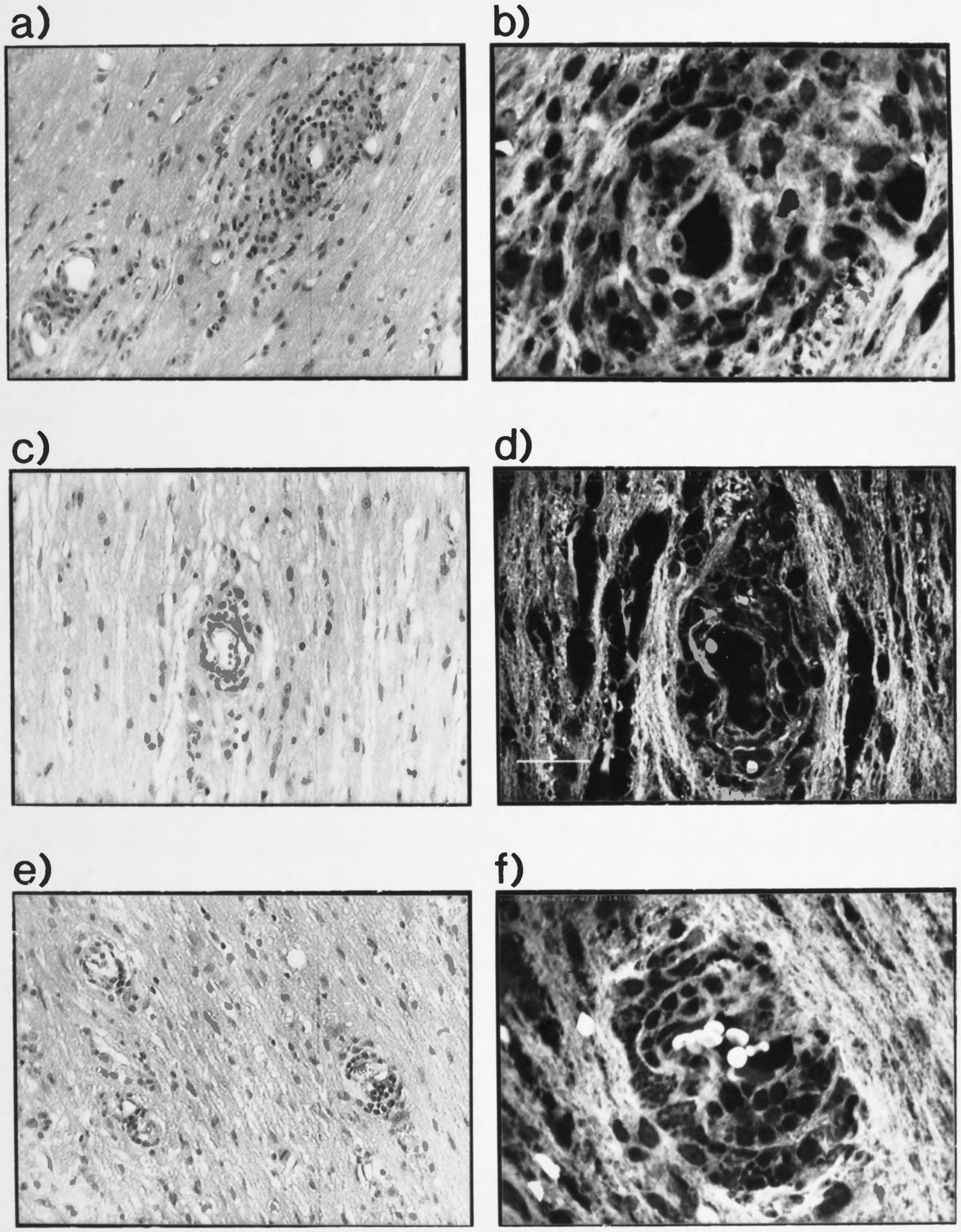


Fig. 3.6: Hematoxylin and eosin stained histological sections from rat spinal cord where recipients received 10^8 H33342 labelled antigen-activated MBP-specific T cells. Perivascular lesions are depicted in sections from the lumbar ((a) and (b)); thoracic ((c) and (d)) and the cervical ((e) and (f)) regions of the spinal cord by conventional light microscopy in (a), (c) and (e) at x225 and by confocal imaging in (b), (d) and (f) at x600. Scale bar corresponds to 25μms.

TABLE 3.1

CELL LINE ACCUMULATION IN THE LOWER SPINAL CORD

Cell line Injected	Recipient	Day 3		Day 4	
		Cell accumulation	Clinical score	Cell accumulation	Clinical score
10^8 fluorescent MBP-specific	Normal	120.0 ± 23.0[a]	2.7 ± 0.3[b]	470.0 ± 330.0	4.7 ± 0.2
10^8 fluorescent OA-specific	Normal	0.3 ± 0.2	0[c]	0.3 ± 0.2	0
10^8 fluorescent OA-specific + 10^8 MBP-specific	Normal	4.7 ± 1.8	0.2 ± 0.2	7.0 ± 0.5	1.5 ± 1.5
10^8 fluorescent MBP-specific + 10^8 OA-specific	Normal	27.0 ± 1.2	0.3 ± 0.3	70.0 ± 36.0	1.3 ± 0.3
10^8 fluorescent MBP-specific	Irradiated	5.3 ± 4.4	0	16.0 ± 4.3	1.5 ± 0.3
10^8 fluorescent OA-specific	Irradiated	0.5 ±0.2	0	ND[d]	ND

[a] represents the number of labelled cells per 100mg wet tissue x10^3 ± se
[b] represents the severity of disease on a scale ranging from 0 to 5 ± se
[c] disease was not evident
[d] not determined

3.7). It was found that an irradiation dose of 1000 rads induced considerable leukopenia, the peripheral blood leukocyte level of irradiated rats being 5-10% and 2-3% of untreated rats when measured at 2 and 3 days post irradiation. The CNS localisation of T cells lines in irradiated animals is shown in Table 3.1. MBP-specific T cell accumulation was less pronounced and occurred 1-2 days later in irradiated than in non-irradiated animals, an observation consistent with the later onset of disease (Table 3.1). Antigen-activated OA-specific T cells did not accumulate in the CNS of irradiated animals (Table 3.1), thus ruling out the possibility of extensive radiation-induced damage to the blood brain barrier allowing non-specific entry of T cells.

As activated MBP-specific cells were found to accumulate to a lesser extent in the spinal cord of irradiated rats than in non-irradiated recipients, an interesting question was whether there were any differences in the size and distribution of inflammatory lesions in the irradiated and non-irradiated animals. To assess histological differences, 3 irradiated and 3 non-irradiated animals were passively transferred with 10^8 MBP-specific cells. Due to the small number of animals observed over two time points, only general observations can be made. One animal from each group was sacrificed when animals were exhibiting the clinical symptom of a flaccid tail. Inflammatory cells were evident in the meningeal (Fig. 3.8 a,b) and perivascular regions (Fig. 3.9 a,b) of the non-irradiated animal. In contrast, in the irradiated animal, lesions were not apparent, but considerable haemorrhage was evident throughout both regions (Figs. 3.8 c,d and 3.9 c,d). In non-irradiated animals, marked perivascular inflammation was evident throughout the spinal cord when animals were exhibiting the symptom of ataxia (Fig. 3.10 a,b,c), whereas in irradiated animals exhibiting similar symptoms, the inflammatory cells which were evident were found to be less organised around the blood vessels (Fig. 3.10 d,e,f) but showed a marked presence of collagen (a component of the extracellular matrix) (Fig. 3.10 e) compared with lesions found in non-irradiated animals (Fig. 3.10 b). Sections from animals exhibiting early signs of EAE (flaccid tail) in the irradiated and non-irradiated groups did not show evidence of astrocytic proliferation (Fig. 3.11 a,c) whereas animals exhibiting more advanced symptoms of disease (ataxia) from both groups showed marked astrocytic proliferation (Fig. 3.11 b,d).

3.3 Discussion

The results presented in this chapter support the hypothesis, originally put forward by Wekerle et al. (1986), that "activated" lymphocytes of any specificity can migrate into the CNS, but that only those cells with specificity for antigens found within the parenchyma of

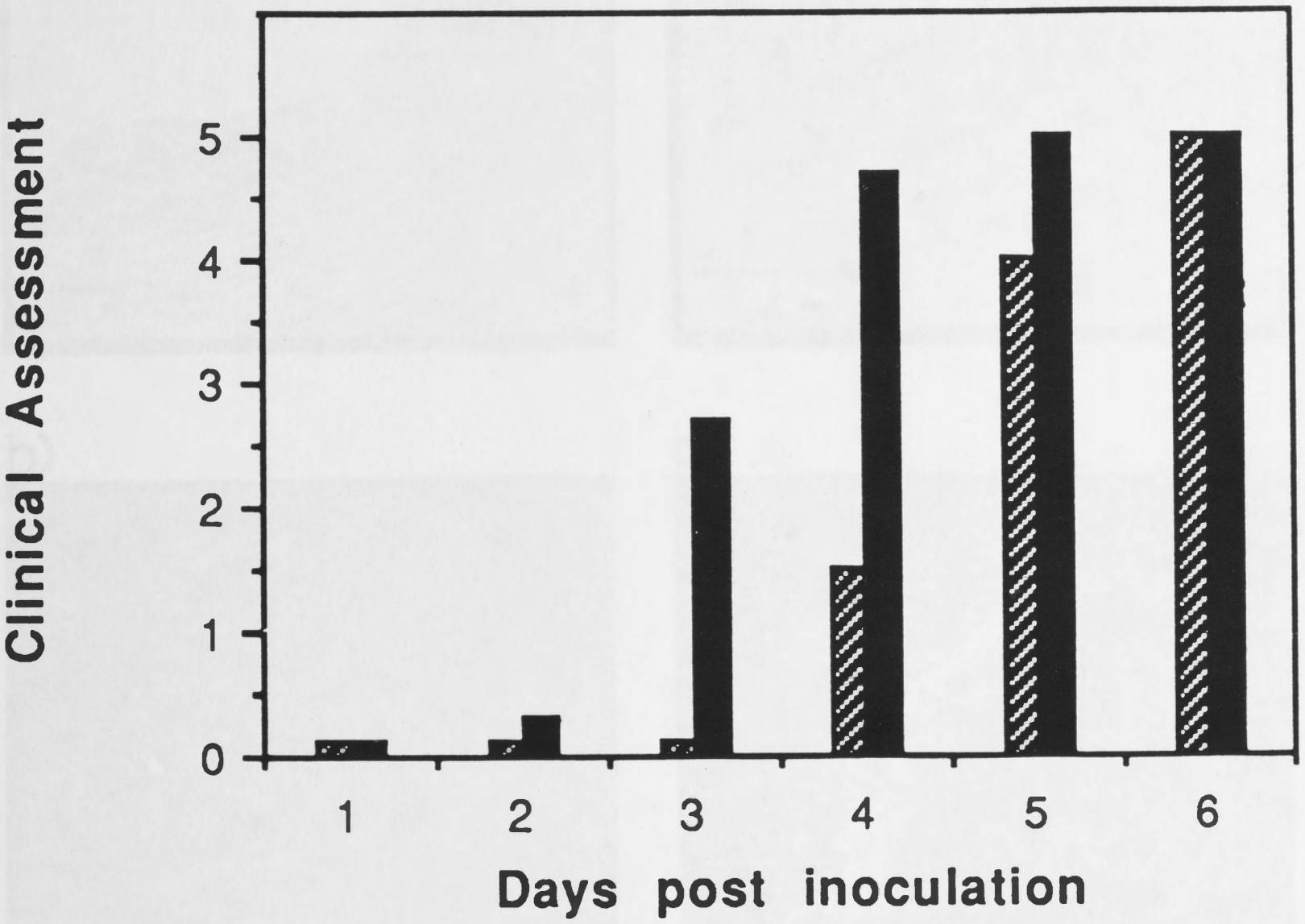


Fig. 3.7: Ability of MBP-specific T cells to cause EAE in irradiated rats. 10^8 H33342 labelled antigen-activated MBP-specific T cells were injected into irradiated (▨) or non-irradiated (■) recipients. Animals were assessed for clinical signs of EAE before being culled for quantification of labelled cells present in the lower spinal cord (Table 3.1). Arbitrary scale of disease severity ranging from 0 to 5 as in Fig. 3.2.

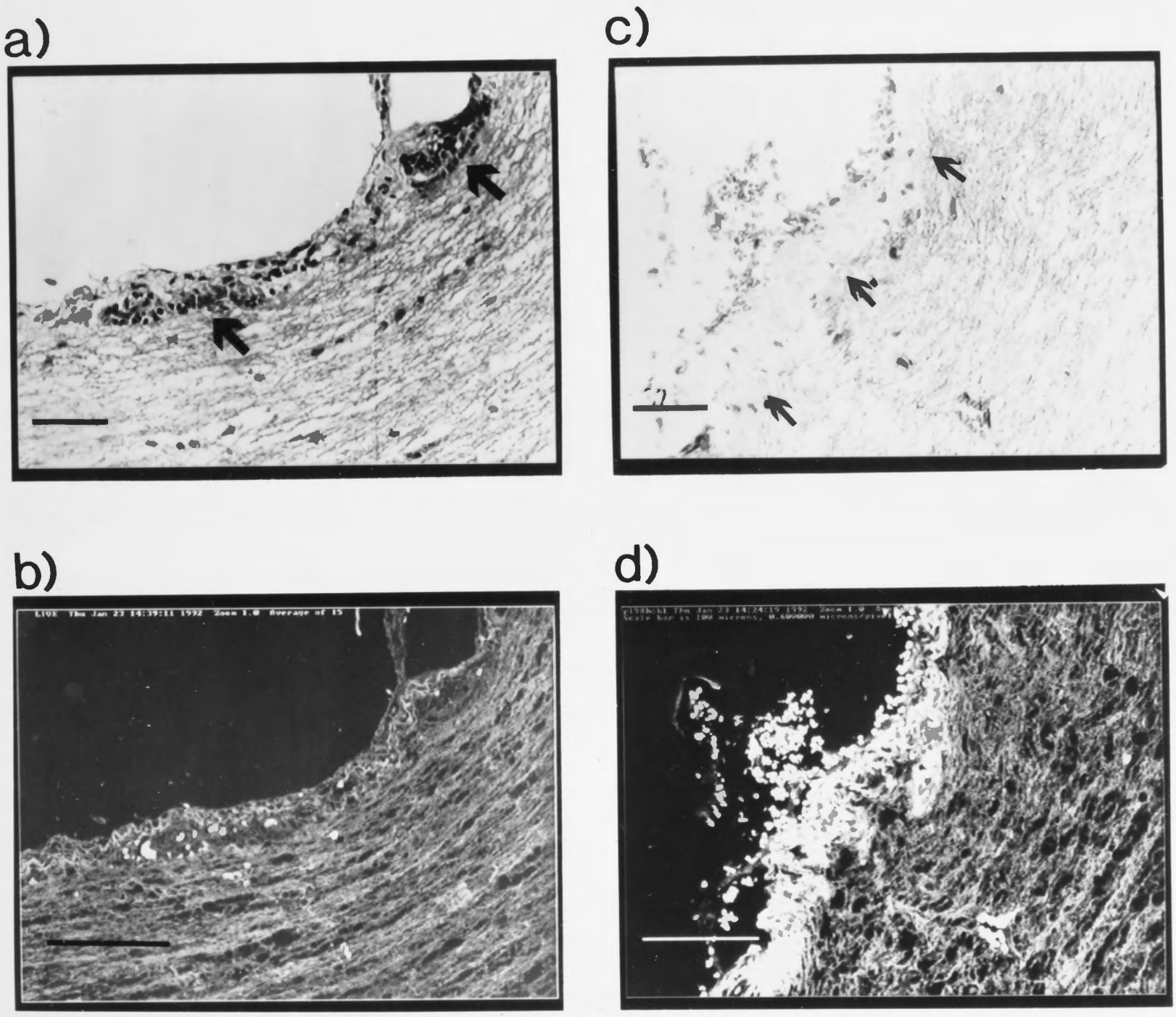


Fig. 3.8: Hematoxylin and eosin stained histological sections of the meningeal regions of rat spinal cord where either non-irradiated (a,b) or irradiated (c,d) recipients received 10^8 H33342 labelled antigen-activated MBP-specific T cells and exhibited early clinical signs of EAE (flaccid tail). Sections were viewed using video imaging at x225 (a,c) (scale bar corresponds to 50μms) and confocal imaging at x200 (b,d) (scale bar corresponds to 100μms). Inflammatory cells (large arrows) are evident in meningeal regions of the spinal cord by light microscopy in a non-irradiated recipient (a), whereas areas of hemorrhage (c, small arrows) are clearly evident in confocal images of an irradiated recipient (d).

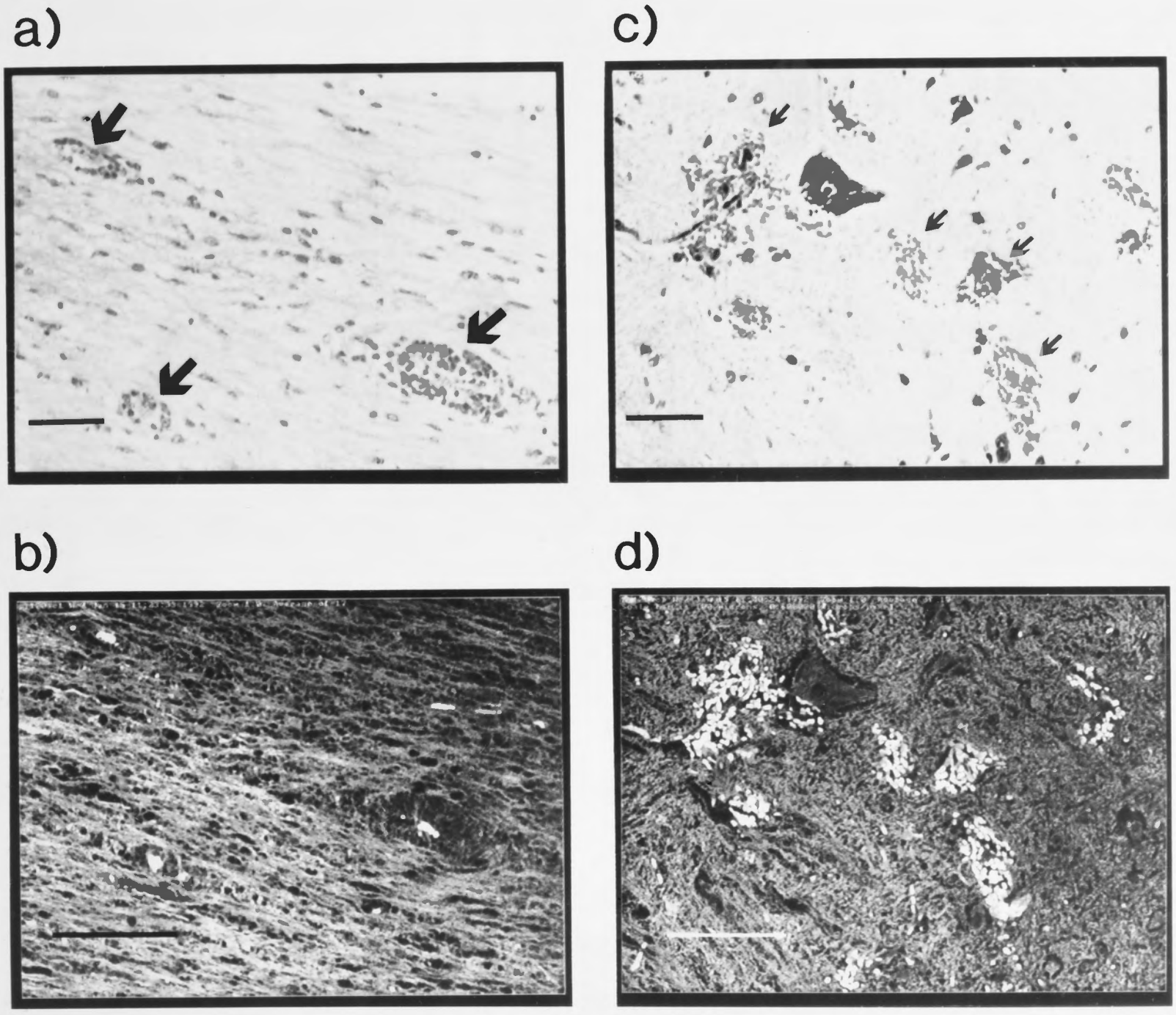


Fig. 3.9: Hematoxylin and eosin stained histological sections of the parenchymal regions of rat spinal cord where either non-irradiated (a,b) or irradiated (c,d) recipients received 10^8 H33342 labelled antigen-activated MBP-specific T cells and exhibited early clinical signs of EAE (flaccid tail). Sections were viewed using video imaging at x225 (a,c) (scale bar corresponds to 50μms) and confocal imaging at x200 (b,d) (scale bar corresponds to 100μms). Inflammatory cells (large arrows) are evident in perivascular regions of the spinal cord by light microscopy in a non-irradiated recipient (a), whereas haemorrhage (c, small arrows) are clearly evident in confocal images of an irradiated recipient (d) .

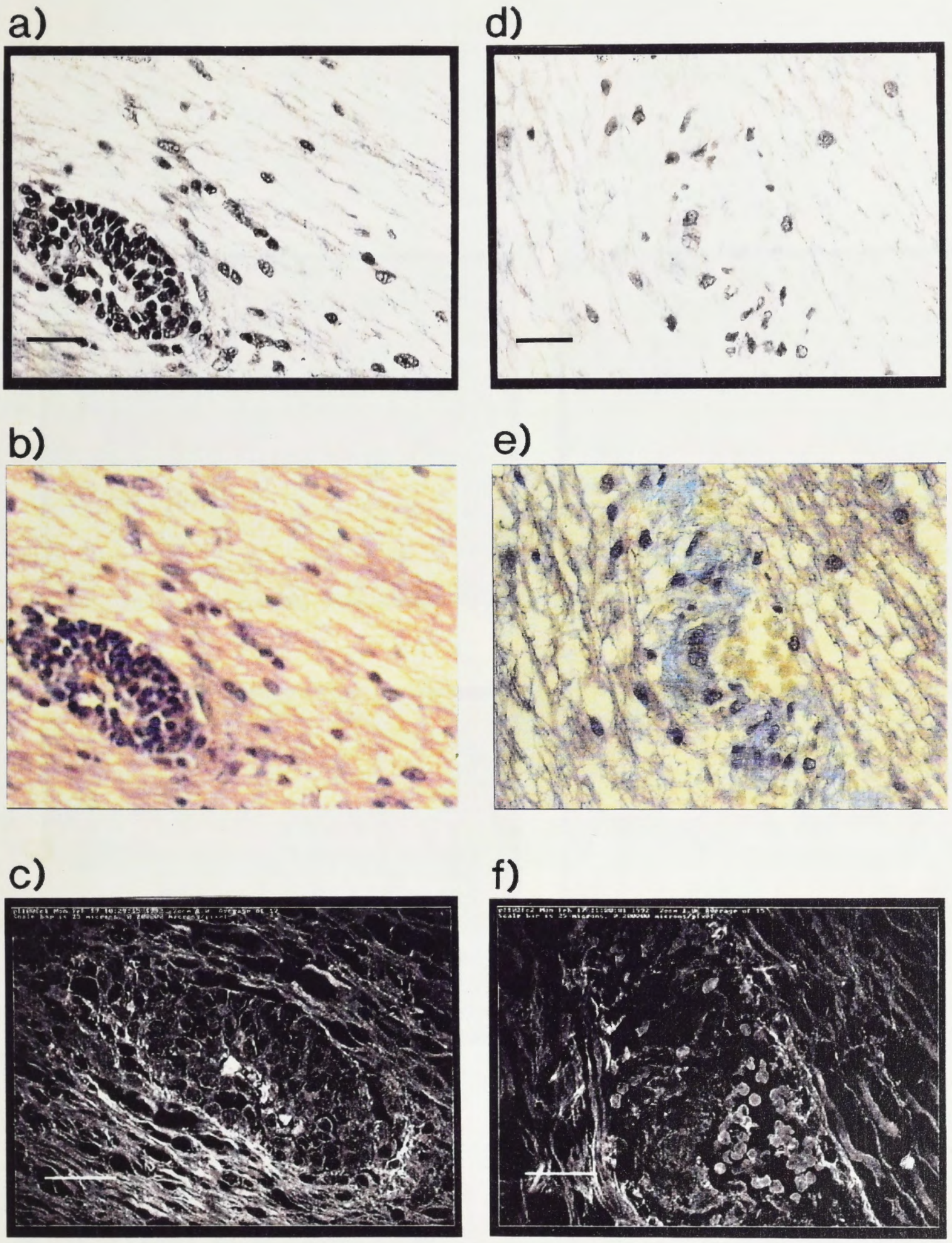


Fig. 3.10: MSB fibrin stained histological sections from rat spinal cord where non-irradiated (a,b,c) and irradiated (d,e,f) recipients received 10^8 H33342 labelled antigen-activated MBP-specific T cells and exhibited advanced clinical signs of EAE (ataxia). Inflammatory cells, presence of collagen (blue area in panel e) and fibrin deposition (fluorescent areas in c and f) are evident in perivascular regions of the spinal cord by light microscopy using black and white (a,d) and colour (b,e) video imaging at x225 (scale bar corresponds to 50μms) and confocal imaging at x600 (c,f) (scale bar corresponds to 25μms).

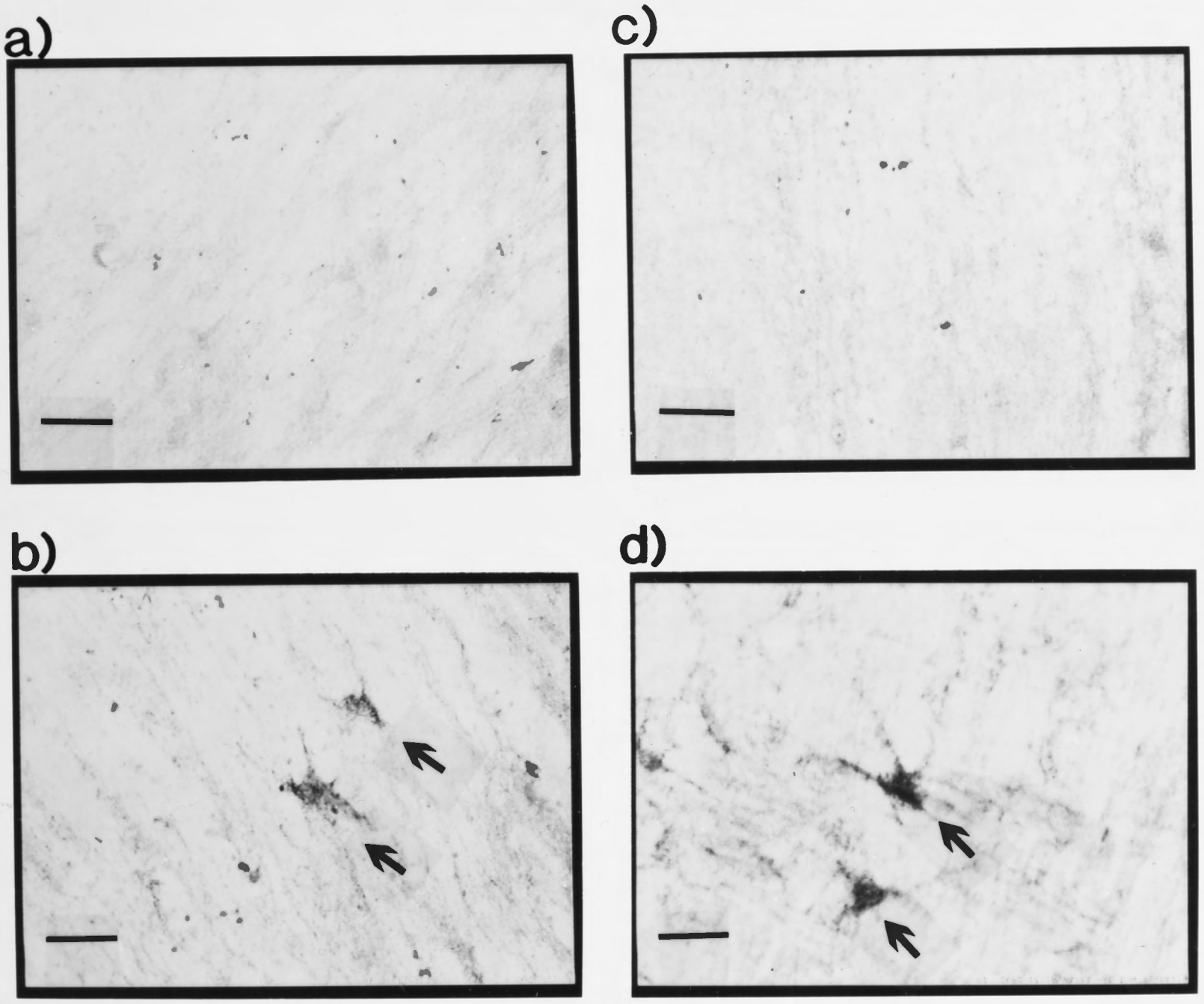


Fig. 3.11: Mercuric chloride (stain for astrocytes) stained histological sections from rat spinal cord where recipients received 10^8 H33342 labelled antigen-activated MBP-specific T cells. Astrocytic proliferation was not evident in the spinal cord of non-irradiated (a) and irradiated (c) recipients exhibiting early signs of EAE (flaccid tail), but was evident (arrows) in non-irradiated (b) and irradiated (d) recipients exhibiting more advanced signs of EAE (ataxia) using video imaging at x450 (scale bar corresponds to 25μms).

the CNS will accumulate. In this study, antigen-activated MBP-, PPD- and OA-specific cell lines all accumulated in the spinal cord to a significant level. The MBP-specific cell line accumulated in an almost exponential fashion whereas the other cell lines although detectable in the CNS for four days did not accumulate. This constant level of cells in the CNS may represent the random ingress and egress of activated CNS and non-CNS antigen specific cells, as suggested by the hypothesis. This view was supported by the observation that IL-2 maintained (non-activated) MBP-specific cells were undetectable in the spinal cord up to 3 days after transfer.

The state of activation of the MBP-specific cells was critical for the induction of disease. Non-activated MBP-specific T cells failed to accumulate in the spinal cord to a substantial degree (Fig. 3.5) and did not cause clinical signs of EAE (Fig. 3.2). These results are consistent with the earlier finding that quiescent cells do not cause disease (Panitch and Ciccone, 1981; Ben-Nun et al., 1981; Holda et al., 1980; Peters and Hinrichs, 1982; Naparstek et al., 1983) and lack the elevated levels of heparanases (Naparstek et al., 1984) which facilitate penetration of the cells through the CNS endothelium and into the inflammatory site (Willenborg and Parish, 1988; Willenborg et al., 1989a, 1989b). Although the non-activated MBP-specific cell line was undetectable in the lower spinal cord on days 1-3 post injection, it did increase to over 10^3 cells per 100 mg tissue on day 4 (Fig. 3.5). This increase on day 4 was also seen in the middle and upper spinal cord, but not to the same extent (Fig. 3.5). This late entrance of non-activated cells could indicate the *in vivo* activation of the cell line by endogenous systemic antigen. Where the antigen is, or in what form is only conjecture, but Day et al. (1978) have reported the existence of a MBP-like serum factor in the serum of normal rats, which could conceivably activate the cells.

Hickey et al. (1991), using immunohistochemical methods, also showed that T lymphoblasts enter the CNS in an apparent random manner while T cells not in an activated state are excluded. While confirming some of these results, the data presented in this chapter differ significantly in two ways. First, activated but non-MBP specific cell lines remained at a constant level in the spinal cord throughout the 4 days period of observation, whereas Hickey et al. (1991) reported that activated non-MBP specific cells left the cord by approximately 2 days. Why the level of activated non-specific cells in the cord returned so rapidly to baseline is unknown. If the number of activated cells in the circulation remained constant (as in the present study) then it would be expected that the cells would be available to move in and out of the spinal cord at an equal rate. Thus, an equilibrium would be maintained, until the cells lost their state of activation. Hickey et al. (1991) did not quantify the number of cells in the blood and other tissues. It is possible that their use of ConA for activation (in contrast to the use of specific antigen in this study) altered the cell surface

properties in such a way so as to ensure their diversion out of the circulation into other tissues. Consequently, the cells would not be available for continual movement in and out of the spinal cord. Secondly, we found that the activated MBP-specific cells accumulated to high levels in the cord with time, while Hickey et al. (1991) showed a peak concentration between 9 and 12 hours after injection with a subsequent decrease and no further accumulation. These results could also be explained by the suggestion that ConA-activated cells are not available in the circulation and therefore unable to accumulate in the spinal cord.

Naparstek et al. (1983) reported that only activated MBP cell lines migrated into the brain and spinal cord. Using ^{51}Cr labelled cells, these investigators found (as in the present study) that the activated MBP specific cells accumulated in the brain and spinal cord with time. Naparstek et al. (1983) reported the disappearance of activated MBP-specific cells from the blood within 1 to 2 days of injection, whereas blood levels remained constant for up to four days post injection in this study. They also reported that significant accumulation in the brain did not occur until day 4, one day before the onset of clinical signs. However, in this study, there was a highly significant accumulation by day two. Further, Naparstek et al. (1983) did not detect the presence of activated non-neurospecific cells in the brain or spinal cord at any time whereas in this study and in that of Hickey et al. (1991) activated non-neurospecific cells were evident in the brain and spinal cord. These differences may reflect the fact that both in this study and in that of Hickey et al. (1991) many more cells (10^8) were used than in the study of Naparstek et al. (1983) (5×10^6).

MBP-specific cellular accumulation was found to be similar throughout the different regions of the spinal cord during the first 3 days post inoculation (Fig. 3.5). This is an unusual finding as EAE is a disease consisting of an ascending paralysis with clinical signs attributed to disturbances of the lumbar-sacral areas of the cord. In this study, it was only at day 4 that there were significantly lower levels of labelled cells in the upper spinal cord than in the other regions (Fig. 3.5). Histologically, however, lesions were apparent throughout the spinal cord at days 4 and 7 post inoculation and there was marked perivascular inflammation evident in all regions of the cord (Fig. 3.6). Possibly lower spinal cord nerve damage is more likely to result in the clinical signs of disease.

The substantial increase in accumulation of the antigen-activated MBP-specific T cells between days one and four is much more than could be accounted for by either random entrance of activated cells or by the replication of the cells *in situ* once they had entered the parenchyma. One possible explanation is that the MBP-specific cell line is being recruited in a non-specific fashion following the initial entrance of specific cells and their subsequent interaction with antigen. If this is the case then one would expect that antigen-activated, non-neuroantigen specific T cells could be recruited into the CNS if injected along with

activated MBP-specific cells. This is indeed what happened (Table 3.1). The CNS accumulation of an OA-activated, OA-specific T cell line increased 15-20 fold when injected simultaneously with a MBP-activated MBP-specific T cell line. However, there was a delay in CNS accumulation of the OA-specific cells (data not shown), suggesting the need to first establish an inflammatory response, and the CNS entry by the OA-specific cells was significantly less than that observed when the MBP-specific T cells were injected alone (Table 3.1). This latter effect may be partly due to a crowding effect as 10^8 non-labelled OA-specific cells added to 10^8 labelled MBP-specific cells had the effect of decreasing the number of labelled cells accumulating in the spinal cord at days 3 and 4 (Table 3.1).

Whereas the results of this study show that the non-specific recruitment of non-neurospecific cells does occur, Naparstek et al., (1983) reported an inability to detect CNS accumulation of non-neuroantigen specific (anti-PPD) T cell lines when they were injected into recipients simultaneously with anti-MBP T cell lines. I have no explanation for this discrepency other than the way the two experiments were done. As stated earlier, in these studies many more cells were used than in the studies by Naparstek et al. (10^8 vs $5x10^6$). Furthermore, donor cell accumulation was assessed, not only in the brain, but in all regions of the spinal cord, where the majority of inflammatory lesions occur. There is no doubt that under these experimental conditions there is significant recruitment of activated non-neurospecific cells into the spinal cord by antigen-activated MBP-specific T cells.

To examine the host contribution to passively induced EAE, the entrance and accumulation of activated MBP-specific cells was assessed in irradiated recipients. Radiation has been reported to have varying effects on the induction of EAE (Levine et al., 1969) with variations probably being related to the species studied, the dose, the timing and the type of radiation. Some investigators found that irradiation of recipients completely inhibited cellular transfer of inflammatory disease (Levine et al., 1969; Werdelin and McCluskey, 1971) and when recipients were restored with bone marrow cells inflammation developed (Werdelin and McCluskey, 1971), whereas others found low dose irradiation (350rads) facilitated induction of EAE with MBP-reactive T cell lines (Zamvil et al., 1985). Sedgewick et al. (1987) reported that regardless of the status of the recipient (irradiated or not), clinical signs were induced when animals received MBP-reactive cells although the number of infiltrating leukocytes was reduced in irradiated animals. This finding may in fact be due to the delayed CNS entry of MBP-specifc T cells. In the present study, activated MBP-specific cells accumulated in the spinal cord of irradiated rats but to a lesser extent than in non-irradiated recipients. There was also a delay in the kinetics of accumulation by about two days which agrees with the altered kinetics of clinical signs (Fig 3.7). Two possible explanations for these results would be that irradiation may directly

affect the endothelium in a way that makes it less adhesive or alternatively the recruitment of "non-specific" host cells may in turn act as recruiting cells in further promotion of the inflammatory response. In this study, the failure of non-specific T cells to accumulate in the CNS rules out the possibility of extensive radiation-induced changes in the vasculature allowing non-specific cellular infiltration.

Differences in the size and distribution of inflammatory lesions in irradiated and non-irradiated recipients were observed by histological methods using spinal cord sections. (Fig. 3.8 to 3.10). In this study, as in that of Sedgewick et al. (1987), the number of inflammatory cells in the spinal cord of irradiated animals was reduced compared to non-irradiated recipient animals. The correlation between the number of lesions in the CNS and flaccid paralysis has been found to be poor (Hoffman et al., 1973; Raine, 1980; Simmons et al., 1982, 1984), and possibly other mechanisms of dysfunction cause clinical symptoms of EAE. Considerable hemorrhage and the exposure of collagen, a component of the extracellular matrix, were found in irradiated recipients in this study, suggesting that the integrity of the vasculature had been affected. Sedgewick et al. (1987) also found that disease in irradiated recipients was associated with substantial hemorrhagic lesions in the spinal cord and brain stem. In addition, a complete absence of hemorrhagic lesions was reported in irradiated recipients receiving OA-reactive cells, suggesting that the vascular damage was caused by the MBP-reactive cells. In support of this hypothesis, the clinical signs of EAE and hemorrhage were preventable by the administration of mAbs specific for the CD4 antigen, leading to the conclusion that the ability to repair damage has been compromised in irradiated animals. In support of this conclusion, it is known that CD4+ cells are cytotoxic to MBP-presenting astrocytes *in vitro* (Sun and Wekerle, 1986) and possibly CD4+ cells cause damage to the blood vessels within the CNS, resulting in the formation of local oedema and astrocytic hypertrophy and consequently in nerve conduction defects and clinical symptoms of EAE.

An important observation in this study was that astrocytic proliferation was evident in irradiated and non-irradiated animals exhibiting advanced clinical signs of EAE (Fig. 3.11). The astrocytic response to injury of the CNS is a process called reactive gliosis (Miller et al., 1986). Scars are formed by astrocytes extending numerous processes that become larger and have a substantial increase in glial filaments (Maxwell and Kruger, 1965; Eng, 1985). The proliferating astrocytes engage in phagocytosis (Noske et al., 1982; Kandel, 1985; Eng, 1985), and consequently astrocytes remove debris and help seal-off damaged brain tissue after neuronal death or injury (Kandel, 1985). This encroachment of astrocytes displaces presynaptic terminals and results in the damaged neurons receiving reduced synaptic input, with the evoked excitatory presynaptic potentials being smaller in

amplitude (Kandel, 1985). Clinical and histological EAE can also be induced in guinea pigs and monkeys by the injection of human glioblastoma (Bigner et al., 1981). These results suggest that astrocytes could be important in the development of clinical signs of EAE with the loss of nerve transmission which results in paralysis possibly being due to astrocytic proliferation slowing the conduction of fibers. The role of astrocytes in EAE will be examined further in Chapter 5.

The question of whether antigen specific lymphocytes accumulate at sites of antigen presence is very important when considering approaches to inhibit autoimmune inflammation. This question has therefore been asked in systems other than EAE. Lightman et al. (1987) reported that T cell lines directed against retinal S antigen (ThS) did not accumulate in the eye of naive recipients to any greater extent than did T cell lines against PPD. This was the case even though recipients of ThS developed autoimmune uveoretinitis. When comparing the studies of Naparstek et al. (1983), Hickey et al. (1991), Lightman et al. (1987) and my own (all of which used T cell lines in a rat model) it is evident that where accumulation of antigen specific cells is reported, the cell lines were activated with specific antigen, but when ConA was used to activate cell lines antigen specific cells did not accumulate. An exception to this is seen in a study of EAE in the mouse by Cross et al. (1990) where MBP activated MBP-specific T cell lines were reported not to accumulate. These investigators used (^{14}C)thymidine-labelled cells and autoradiography to identify and localize cells. This technique would seem to be less sensitive as no cells were seen until day 5 post transfer, and thus it is difficult to compare our results with theirs. The question of whether the type of activation (specific antigen vs ConA) influences the ability of neurospecific cell lines to accumulate needs to be addressed.

Both activated non-neurospecific cell lines (OA- and PPD-specific) enter the lower spinal cord and persist at a low level. There was a difference in the absolute numbers of cells observed for the two cell lines, with the PPD-specific cells being more numerous. One possible explanation for this difference is that PPD-specific T cells show some cross reaction with MBP and the cell line is therefore behaving as a neuroantigen-specific cell line. Cross-reactivity between PPD and MBP has been described previously by Vandenbark et al., (1975). This explanation would seem unlikely, however, because if the cells were reacting with antigen, a higher level of PPD cells in the spinal cord with accumulation with time would be expected. This is clearly not the case. Furthermore, PPD-specific cells were not more numerous than OA-specific cells in other regions of the spinal cord (Fig. 3.5). Another possibility is that the two cell lines differed in their degree of "activation" and hence their ability to enter the CNS parenchyma. This brings up the question of what exactly "activation" represents. This is currently unknown but it has been suggested that activation

translates to the expression of enzymes on the lymphocyte surface that facilitate the degradation of the sub-endothelial basement membrane and extracellular matrix thus promoting emigration (Willenborg and Parish, 1988; Willenborg et al., 1989a, 1989b). There are of course a number of other interpretations of what "activation" represents such as the up-regulation of lymphocyte function associated antigens (LFA-1) with increased adhesion to endothelial cells (Cannella et al., 1990; Sobel et al., 1990), or the ability of activated but not non-activated cells to produce a given range of cytokines (Powell et al., 1990). Comparing specific antigen-activated with ConA-activated cells with respect to their ability to express enzymes, adhesion molecules or various cytokines may provide valuable information.

In conclusion, this study demonstrates that activated lymphocytes of any specificity enter the spinal cord whereas only neuro-antigen specific cells accumulate, initiating an inflammatory response with resultant non-specific recruitment of mononuclear cells and associated vascular damage. Non-activated cells, even those with neural antigen specificity fail to enter the cord. Understanding the nature of what an "activated" lymphocyte is will possibly enable the design of strategies to inhibit such immune-mediated inflammation.

3.4 Summary

Using EAE in the rat as a model of CNS inflammation, activated and quiescent T lymphocytes with different antigen specificities were labelled with the fluorescent dye Hoechst 33342 and tested by fluorescence microscopy for their ability to accumulate in different regions of the spinal cord and in other organs at varying times post inoculation. With this highly sensitive assay it was found that activated MBP-specific T cell lines accumulated in the spinal cord (a 1000fold increase in the lumbar/sacral region by day 4) and caused clinical signs of EAE. In contrast, IL-2 maintained (quiescent) MBP-specific T cell lines failed to accumulate in the CNS and cause disease. Activated OA-specific and PPD-specific T cell lines were also found at significantly higher levels in the spinal cord than non-activated cells although they failed to accumulate to a substantial degree when injected alone. When injected with activated MBP-specific T cells the activated OA-, and PPD-specific cell lines accumulated in the spinal cord following initial accumulation of the MBP-specific cells, demonstrating that during the inflammatory process there is considerable non-specific recruitment of cells into the inflammatory site. CNS accumulation of activated MBP-specific T cell lines occurred 1-2 days later in irradiated animals than in non-irradiated recipients. This was consistent with irradiated animals also exhibiting a later onset of disease and suggests that irradiation may directly affect the endothelium in a way

that makes it less adhesive. Preliminary observations also supported the view that in irradiated animals tissue repair mechanisms in the CNS are compromised. Considerable hemorrhage and the exposure of collagen, a component of the extracellular matrix, were found in irradiated recipients in this study, suggesting that the integrity of the vasculature had been affected. An important observation in this study was that astrocytic proliferation was evident in irradiated and non-irradiated animals exhibiting advanced clinical signs of EAE. These results suggest that astrocytes could be important in the development of clinical signs of EAE. In conclusion, this study demonstrates that activated lymphocytes of any specificity enter the spinal cord, and that the neuro-antigen specific cells accumulate there and lead to the recruitment of other cells. Non-activated cells, even those with neural antigen specificity fail to enter the spinal cord. Understanding the nature of what an "activated" lymphocyte is may allow the design of strategies to inhibit such immune mediated inflammation.

CHAPTER 4: AGE RELATED CHANGES IN THE SEVERITY AND CHRONICITY OF EAE

4.1 Introduction

Ageing is associated with a marked deterioration of the immune system (Gardner, 1980; reviewed by Talor and Rose, 1991) and a significant increase in the incidence of autoimmune diseases (Talor and Rose, 1991). Two functional changes in the immune system which are thought to be associated with the increased incidence of infectious and autoimmune diseases observed in the elderly are the decline in the immune response to exogenous stimuli and a loss of self tolerance (Ackerman et al., 1991).

One of the anatomically most prominant age-related changes is the decline in size of the thymus (Boyd, 1932) and the decline in immune responsiveness with age has been demonstrated most prominantly in T-cell-mediated immunity (reviews by Kay, 1980; Kay and Makinodan, 1981; Nagel, 1983; and Ackerman et al., 1991). Age-related alterations in lymphocyte function include a decrease in CD4+ and CD8+ T cell activity, a decrease in T cell response to mitogens and antigens and a decrease in the production of lymphokines including IL-2 (reviewed by Ackerman et al., 1991). Age-related changes in macrophages and B cell function (Heidrick and Makinodan, 1972; Gardner and Remington, 1978; Becker et al., 1981; Goidl et al., 1983; reviewed by Ackerman et al., 1991) may also indirectly influence T-cell-mediated responses.

MS and its animal model EAE are both age dependent diseases. It is rare for children, or adults after the age of 50, to develop MS (Kurland, 1952). The presence of a mature lymphoid system is necessary for the development of EAE (Paterson et al., 1970) as neonatal rats are resistent to EAE and become susceptible at approximately 8 weeks of age. EAE is also a T cell mediated disease, as demonstrated by an inability of genetically susceptible animals depleted of T cell precursors by neonatal thymectomy (Wick, 1972; Bernard et al., 1976), thoracic duct drainage (Gonatas and Howard, 1974), or anti-thymocyte serum (Ortiz-Ortiz and Wiegle, 1976) to manifest symptoms. Injection of normal thymocytes restores susceptibility to disease (Arnason et al.,1962; Lennon and Byrd, 1973; Ortiz-Ortiz and Wiegle, 1976; Gonatas and Howard, 1979). The important role played by T cells in this process is further illustrated by the demonstration that the adoptive transfer of sensitised T cells from an EAE donor (Paterson, 1960; Stone, 1961; Astrom and Waksman, 1962; Levine et al., 1970; Bernard et al.,1976) or the introduction of encephalitogenic T cell clones into naive syngeneic recipients, cause disease symptoms (Ben-Nun et al., 1981; Hauser et al., 1984b; Raine, 1984). The course of the disease can also be modified by immunosuppressive medication (reviewed by Raine, 1985), such as treatment with anti-class II antibodies or treatment with anti-CD4 antibodies. Thus, it is clearly established that EAE is mediated by

MHC class II antigen-restricted T cells (Pettinelli and McFarlin, 1981; Zamvil et al., 1985). However, the actual mechanisms initiating the CNS entry of T cells and the subsequent CNS damage produced by the ensuing inflammatory response remain unclear. In this context, it is of considerable interest to determine whether advanced age, which is associated with a decline in T cell-mediated immunity, protects against EAE, a T cell mediated disease.

Conflicting results have been found in previous studies on EAE development in aged animals. Mc Farlin et al. (1974) reported recurrent EAE in aged (6 months old) female Lewis rats, whereas young rats of the same strain exhibited a monophasic course of disease. Ben-Nun et al. (1981) found Lewis rats developed recurrent or chronic EAE when sensitised with MBP at an advanced age. In old mice, susceptibility to EAE induced with bovine proteolipid apoprotein (PLP) and MBP was found to be reduced significantly although some old mice develop histologic EAE without clinical signs. Lymphocyte proliferative response to mitogens and antigens, and IL-2 production were also decreased in aged mice (Endoh et al., 1990). It was subsequently found that the reduced susceptibility of old mice to EAE and the decreased T cell functions cannot be restored by the treatment of old mice with thymic hormones (Endoh and Tabira, 1990). However, Endoh et al. (1990) found that clinical signs and histological lesions were more severe in the small number of aged mice which did develop disease, indicating that although there was a decrease in susceptibility to EAE with age, when disease was apparent in aged animals it was severe. Levine and Sowinski (1976) demonstrated an age related decline in susceptibility to EAE in rats. They found that with intense immunisation (MBP+CFA+pertussis vaccine) of rats (inbred Fischer 344 (F344) males) aged 2, 6 and 12 months, EAE developed at a similar rate and severity. There was a lesser response in 18 and 24 month olds with a more protracted incubation period and milder or absent signs. However, with less intense immunisation (without pertussis or 1μg MBP) almost all 6 month olds developed disease but not a single 24 month old developed EAE under these conditions. The data indicated an age related decline in susceptibility to EAE although histologic examination of inoculated feet and draining lymph nodes revealed no deficiencies in the processing of the antigenic inoculum. However, none of their aged rats seemed to be free of illness and extensive leukemic infiltration of lymphoid organs might have immunosuppressed recipients. Levine and Sowinski (1976) suggested that because of the multiplicity of specific illness found in F344 rats (Sass et al., 1975), that this was not a good model for assessing age related factors and possibly a strain with a longer life-span would be a better model.

In this study, the pathogenesis and immunoregulation of EAE was investigated by specifically looking at age related differences in Lewis (JC) rats, a rat strain which does not suffer from a multiplicity of age-related illnesses. In a preliminary experiment, old rats (over one year old) were found to have an increased chronicity of the disease compared to young rats when actively induced with EAE. Consequently, a combined clinical, histopathological

and biochemical study of EAE in Lewis (JC) rats of differing ages was performed to understand the clinical manifestations of this neurological disease. The specific aims of the study were:

1) to establish clinical and histopathological changes in the disease of aged animals, to examine sex-related differences in disease severity in aged animals and to determine the mechanisms of recovery;
2) to determine whether advanced age protects against EAE as it does against MS in humans;
3) to correlate the clinical and histological changes and disease recovery with the production of MBP-specific antibody, corticosteroids, TNF, nitric oxide and prostaglandins (see Chapter 6).

4.2 Results

4.2.1 Age-related differences in clinical profiles

EAE was actively induced by immunisation with MBP in CFA in rats of different ages to determine the age-related differences in the development of disease. Representative clinical disease profiles of male geriatric (24-26 months old), middle-aged (12-13 months old) and young (2-3 months old) rats are shown in Fig. 4.1. The clinical course of the disease was typical for this model of EAE, namely animals initially developed a flaccid tail followed by hindlimb weakness and then paralysis. The severity of disease was similar in that paralysis was evident in all age groups, however the disease chronicity in older animals was significantly higher. The number of deaths was high in geriatric rats and a chronic relapsing course with a high number of deaths also occurred in middle-aged rats (Fig. 4.1). It is evident in Table 4.1 that young animals exhibited a short monophasic episode of hindlimb paralysis (1.7 days), they recovered from this episode of paralysis with no obvious deficit, had a low death rate (10%), a short length of disease (7.4 days) and only 11% of animals exhibited a mild second episode of disease. In comparison, there was a slower and less vigorous response initially in middle-aged and geriatric rats which have a mean day of onset 2 to 3 days later than young recipients. Middle-aged animals had a high death rate (35%) and high relapse rate (62%) with significantly more days paralysed (4.6) and a protracted length of disease was also evident (18.6 days). In geriatric rats, there was a higher death rate (54%) but, animals that didn't die had less chronic disease (2.9 days with hindlimb paralysis and length of disease of 12.3 days) with no evidence of relapses. These data clearly indicate an age-related increase in the disease chronicity of EAE in middle-aged and geriatric rats with significantly more deaths evident in both middle-aged and geriatric animals than in young animals.

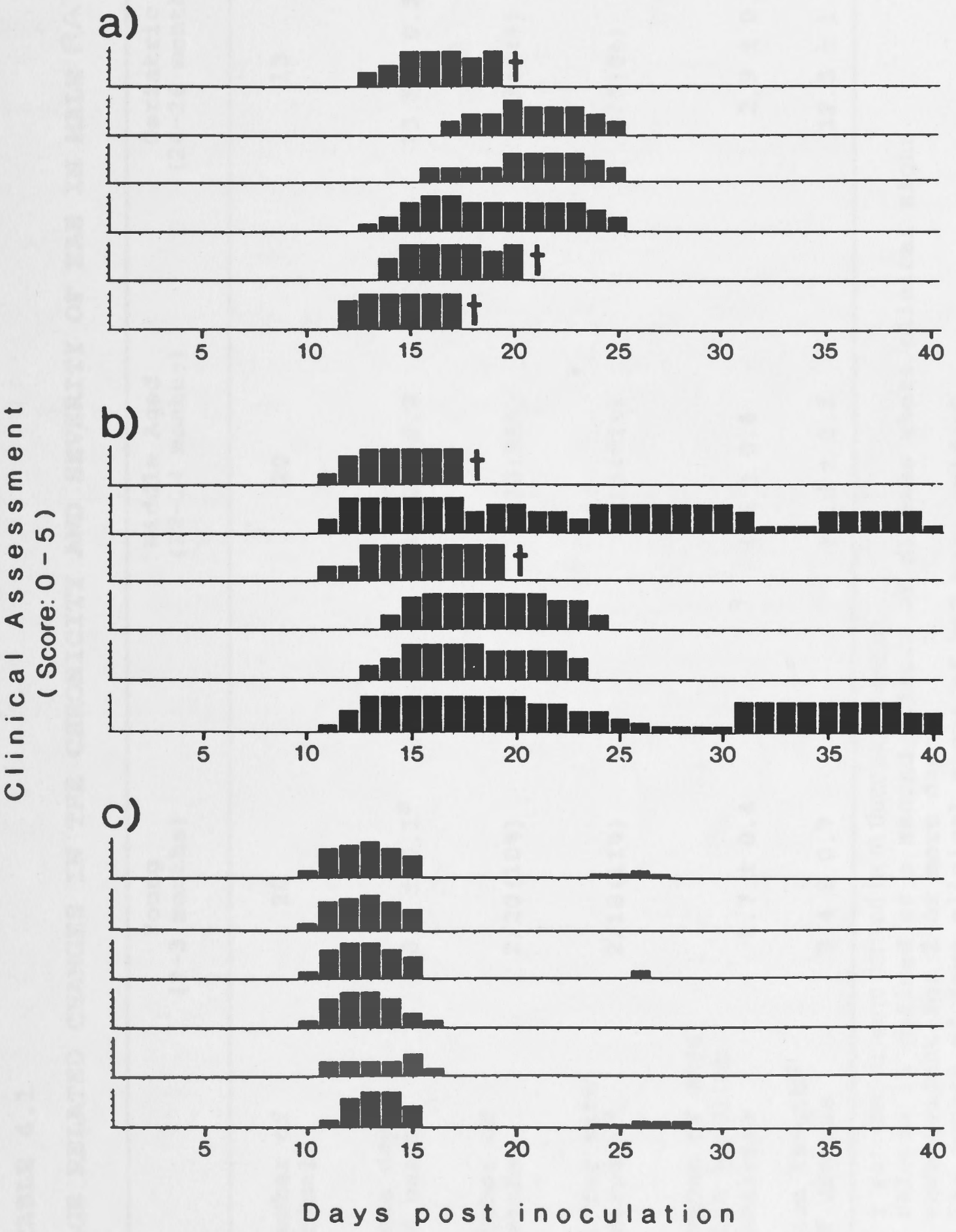


Fig. 4.1: Effect of age on actively induced EAE. Representative clinical profiles of male geriatric (a), middle-aged (b) and young (c) rats inoculated with 50µg MBP in CFA containing 400µg *M. butyricum.* Animals were assessed for clinical signs of EAE daily and an arbitrary scale of disease severity ranging from 0 to 5 was used as in Fig. 3.2. Each line represents the clinical course of one rat.

TABLE 4.1

AGE RELATED CHANGES IN THE CHRONICITY AND SEVERITY OF EAE IN MALE RATS.

	Young (2-3 months)	Middle Aged (12-14 months)	Geriatric (24-26 months)
Number of animals	20	20	13
Mean day of onset	10.5 ± 0.1[a]	12.0 ± 0.3	13.5 ± 0.5
Number of deaths	2/20(10%)	7/20(35%)	7/13(54%)
Number with relapses[b]	2/18(11%)	8/13(62%)	0/6(0%)
Number of days with hindlimb paralysis	1.7 ± 0.4	4.6 ± 0.8	2.9 ± 0.7
Mean length[c] of disease	7.4 ± 0.7	18.6 ± 2.2	12.3 ± 1.3

[a] ± se (Resultswere derived from three experiments)
[b] relapse is defined as a second episode of disease where clinical signs were evident for 2 or more days
[c] the number of days clinical signs of EAE were evident

A general observation made during these experiments was that there were slight variations found in the course of disease between experiments. In Fig. 4.2, it is evident that in one experiment, middle-aged (a) and young (c) rats had more relapses than in a second experiment where middle-aged (b) and young rats (d) received a different batch of MBP, although the MBP had been prepared using the same method. However, in this second experiment middle-aged rats exhibited a higher death rate. It has been suggested previously (Mc Farlin et al., 1973; Martenson et al., 1974) that the initial attack of EAE may be produced by one encephalitogenic determinant of MBP whereas the second attack is related to a different determinant. These results are consistent with this hypothesis and consequently one substantial batch of MBP was prepared and used for the remainder of the experiments. Minor variations may also be attributed to seasonal and individual differences.

4.2.2 Sex-related differences in clinical profiles

EAE was actively induced by immunisation with MBP in CFA in male and female rats of different ages to determine the sex-related differences in the development of disease. In Fig. 4.3, representative clinical profiles of geriatric male (a) and female (b), middle-aged male (c) and female (d) and young male (e) and female (f) rats are shown. Disease severity and the number of deaths was highest in geriatric rats of both sexes (a,b), a chronic relapsing course with significant deaths occurred in both male and female middle-aged rats (c,d), whereas a typically monophasic disease was evident in both male and female young rats (e,f). It is evident from Fig. 4.3 and pooled data in Table 4.2, that males generally exhibited increased severity and chronicity compared with females of the same age. Thus, it was found that at all ages, females had a mean day of onset approximately 2 days after males of the same age, and fewer deaths, less numbers of days with paralysis with generally a shorter duration of disease.

To test whether hormonal levels were contributing to the generally less severe and less chronic form of the disease found in females, middle-aged sterilised females were compared with non-sterilised females of the same age using both actively and passively induced disease models of EAE. In Table 4.3, it is evident that no significant differences between sterilised and control animals were found in these experiments. The sterilisation of females had no detrimental effect on the onset or severity of clinical EAE in middle-aged animals using either active or passive induction of the disease.

4.2.3 The Role of MBP-specific antibody

To assess whether MBP-specific antibody production correlated with the onset, severity or chronicity of EAE, the serum anti-MBP antibody titres of young and middle aged male rats were determined up to 80 days post inoculation with MBP in CFA (Fig. 4.4). In this study, anti-MBP antibody levels were found to reach a peak by day 30 post inoculation

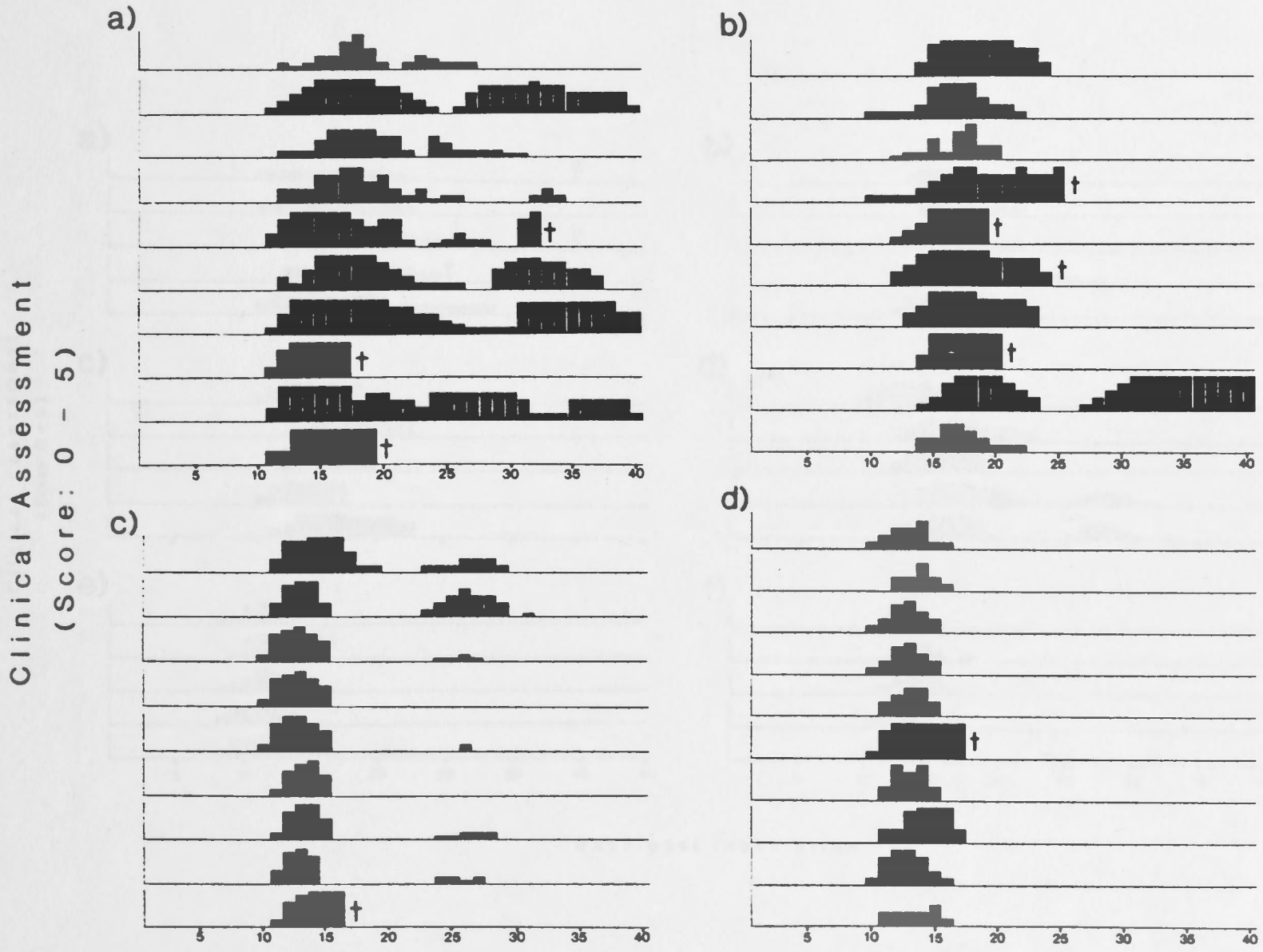


Fig. 4.2: Effect of different batches of MBP on the age-dependent progression of disease. Clinical profiles of middle-aged (a,b) and young (c,d) male rats inoculated with different batches (a,c and b,d) of MBP (50µg) in CFA containing 400µg *M. butyricum*. Animals wer assessed for clinical signs of EAE daily and an arbitrary scale of disease severity ranging fro 0 to 5 was used as in Fig. 3.2.

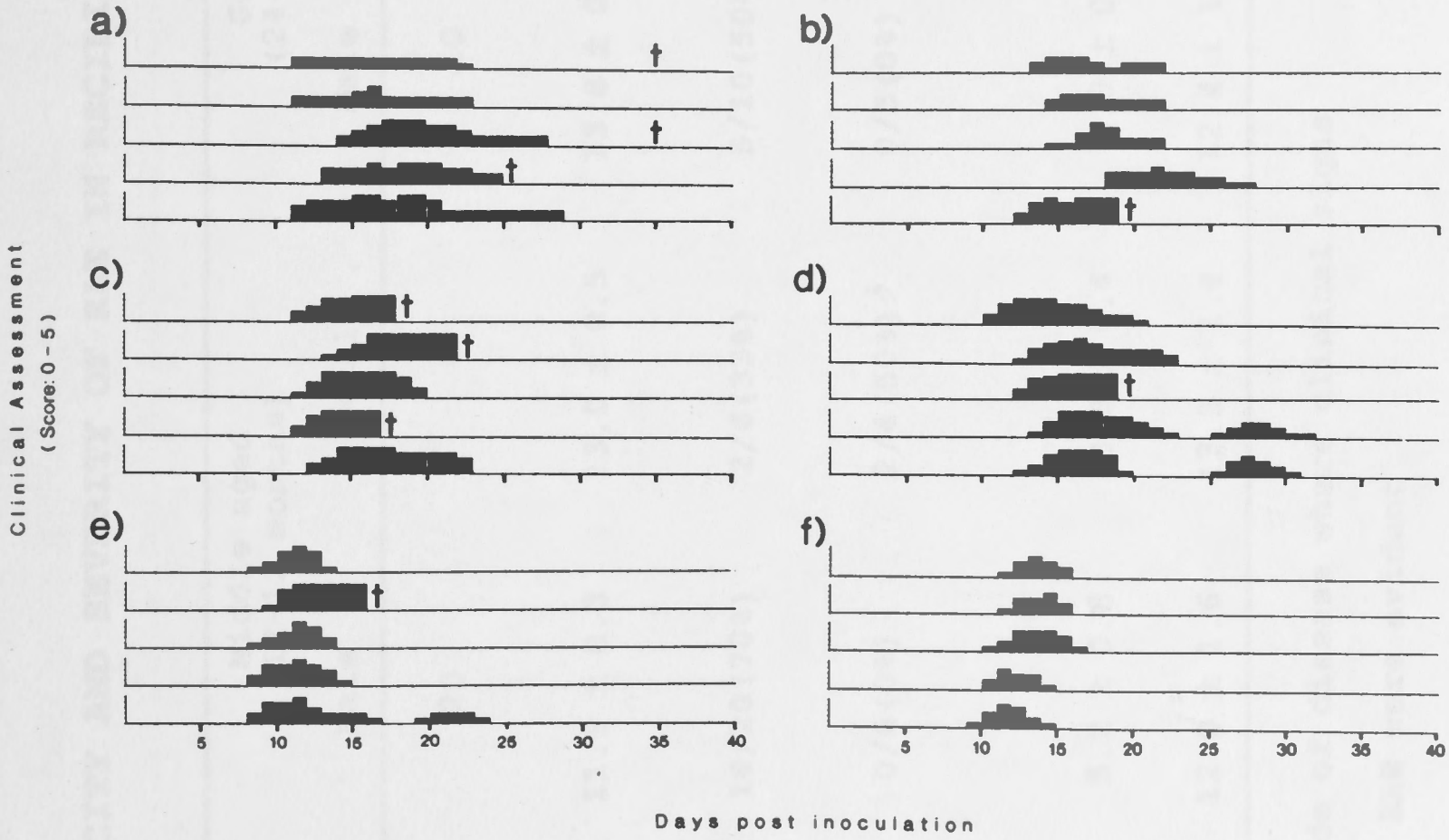


Fig. 4.3: Relationship of age and sex on actively induced EAE. Representative clinical profiles of geriatric male (a) and female (b), middle-aged male (c) and female (d) and young male (e) and female (f) rats inoculated with 50µg MBP in CFA containing 400µg *M. butyricum*. Animals were assessed for clinical signs of EAE daily and an arbitrary scale of disease severity ranging from 0 to 5 was used as in Fig. 3.2.

TABLE 4.2

SEX RELATED CHANGES IN THE CHRONICITY AND SEVERITY OF EAE IN RECIPIENTS OF DIFFERENT AGES

	Young (2-3 months)		Middle aged (12-13 months)		Geriatric (24-26 months)	
	Male	Female	Male	Female	Male	Female
Number of Animals	24	23	20	6	10	8
Mean day of onset	9.5 ± 0.1[a]	11.3 ± 0.3	11.9 ± 0.3	13.0 ± 0.5	13.8 ± 0.5	16.1 ± 0.8
Number of deaths	5/24(21%)	0/23(0%)	14/20(70%)	2/6(33%)	5/10(50%)	2/8(25%)
Number of relapses[b]	3/19(16%)	2/23(9%)	0/6(0%)	2/4(50%)	0/5(0%)	0/6(0%)
Number of days with hindlimb paralysis	1.8 ± 0.3	0.4 ± 0.2	5.2 ± 0.8	3.8 ± 0.4	1.9 ± 0.6	0.75 ± 0.5
Mean length[c] of disease	7.7 ± 0.5	6.2 ± 0.3	12.0 ± 1.6	13.3 ± 1.4	12.4 ± 1.6	9.5 ± 1.3

[a] ± se
[b] relapse is defined as a second episode of disease where clinical signs were evident for 2 or more days
[c] the number of days clinical signs of EAE were evident

TABLE 4.3

CHRONICITY AND SEVERITY OF EAE IN STERILISED AND NON-STERILISED MIDDLE AGED FEMALE RECIPIENTS

	Actively Induced EAE (MBA + CFA)		Passively Induced EAE ($50x10^6$ ConA-activated effector cells)	
	Sterilised	Non-Sterilised	Sterilised	Non-Sterilised
Number of Animals	5	5	4	5
Mean day of onset	12.0 ± 0.3[a]	12.0 ± 0.3	4.0 ± 0.4	4.6 ± 0.2
Number of deaths	0/5(0%)	0/5(0%)	0/4(0%)	0/5(0%)
Number of relapses[c]	2/5(40%)	0/5(0%)	0/4(0%)	0/5(0%)
Number of days with hindlimb paralysis	5.3 ± 0.3	7.7 ± 0.3	0.8 ± 0.3	0.2 ± 0.2
Mean length[d] of disease	18.3 ± 6.5	23.3 ± 4.7	14 ± 1.8	11.8 ± 1.9

[a] memory animals received $50x10^6$ ConA activated effector cells 4 days postnatally
[b] ± se
[c] relapse is defined as a second episode of disease where clinical signs were evident for 2 or more days
[d] the number of days clinical signs of EAE were evident

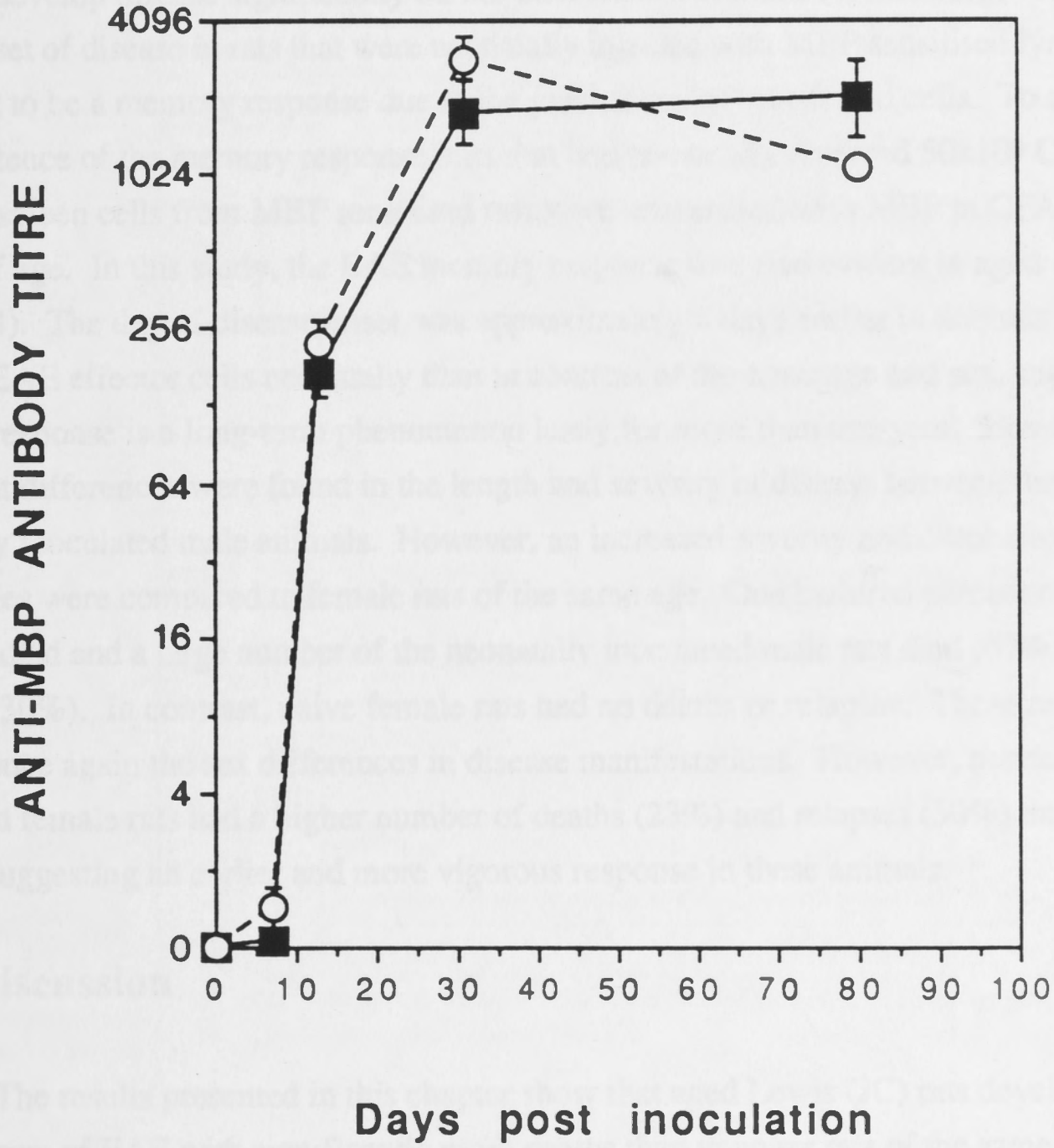


Fig. 4.4: Serum anti-MBP antibody titres of young (■) and middle aged (○) male rats inoculated with 50µg MBP in CFA containing 400µg *M. butyricum*. Antibody titres are expressed as the reciprocal of the serum dilution. Each data point represents the mean and standard error of 10 animals.

and when measured at 80 days post inoculation were found to be still at the same high level. No significant differences were found between the anti-MBP antibody titres of young and middle-aged animals.

4.2.4 Memory response in aged animals

Neonatal rats, passively transferred with ConA-activated spleen cells from MBP-immunised rats do not show clinical or histopathological evidence of disease after the transfer of cells. If these animals are actively sensitised with MBP in CFA at 10 to 12 weeks of age, they will develop disease significantly earlier than control animals (Willenborg, 1986). The earlier onset of disease in rats that were neonatally injected with MBP sensitised lymphocytes is thought to be a memory response due to the persistence of transferred cells. To ascertain the persistence of the memory response, rats that had neonatally received $50x10^6$ ConA-activated spleen cells from MBP sensitised rats were immunised with MBP in CFA at 13 months of age. In this study, the EAE memory response was also evident in aged animals (Table 4.4). The day of disease onset was approximately 2 days earlier in animals which had received EAE effector cells neonatally than in controls of the same age and sex, suggesting the memory response is a long-term phenomenon lastly for more than one year. However, no significant differences were found in the length and severity of disease between the naive and neonatally inoculated male animals. However, an increased severity and chronicity was found when males were compared to female rats of the same age. One hundred percent of the naive male rats died and a large number of the neonatally inoculated male rats died (67%) or had relapses (30%). In contrast, naive female rats had no deaths or relapses. These results confirm once again the sex differences in disease manifestations. However, neonatally inoculated female rats had a higher number of deaths (23%) and relapses (50%) than naive females suggesting an earlier and more vigorous response in these animals.

4.3 Discussion

The results presented in this chapter show that aged Lewis (JC) rats develop a more chronic form of EAE with significantly more deaths than younger rats of the same strain. Although there was a slower and less vigorous response initially in aged animals, the disease chronicity in middle-aged and geriatric rats was significantly higher. Young animals exhibited a short monophasic episode of disease, had a low death rate and only a few animals exhibited a mild second episode of disease. These results suggest there is a deficit in the resolution of paralysis resulting from CNS inflammatory lesions in older animals. The study also revealed that male rats exhibited increased severity and chronicity of disease compared with females of the same age. However, sterilisation of females did not have a significant effect on disease profiles.

TABLE 4.4

DAY OF ONSET, CHRONICITY AND SEVERITY OF EAE IN MIDDLE-AGED NAIVE AND PREVIOUSLY INOCULATED RECIPIENTS.

	Naive		Memory[a]	
	Male	Female	Male	Female
Number of Animals	10	9	9	13
Mean day of onset	10.6 ± 0.3[b]	11.6 ± 0.2	8.4 ± 0.2	8.9 ± 0.2
Number of deaths	10/10(100%)	0/9(0%)	6/9(67%)	3/13(23%)
Number of relapses[c]	0/0(0%)	0/9(0%)	1/3(33%)	5/10(50%)
Number of days with hindlimb paralysis	6.7 ± 0.8	2.7 ± 1.0	6.3 ± 1.1	3.1 ± 0.9
Mean length[d] of disease	10.1 ± 1.1	12.7 ± 1.0	11.0 ± 1.8	11.8 ± 1.9

[a] memory animals received $50x10^6$ ConA activated effector cells 4 days postnatally
[b] ± se
[c] relapse is defined as a second episode of disease where clinical signs were evident for 2 or more days
[d] the number of days clinical signs of EAE were evident

Although EAE is usually a monophasic self-limiting disease in rats, recurrent EAE has been previously reported in aged (6 months old, Mc Farlin et al., 1974 and 13 months old, Ben-Nun et al., 1980) female Lewis rats. Kallen and Logdberg (1982) also reported there was an age-effect on the susceptibility of Lew/Mol (a nearly EAE resistant Lewis substrain) rats. In contrast, an age related decline in susceptibility to EAE has been demonstrated by some researchers (Levine and Sowinski, 1976; Endoh et al., 1990). Levine and Sowinski (1976) found that with intense immunisation (MBP+CFA+pertussis vaccine) of rats (inbred Fischer 344 males) aged 2, 6 and 12 months, EAE developed at a similar rate and severity. However, there was a lesser response in 18 and 24 month olds with a more protracted incubation period and milder or absent signs. With less intense immunisation (without pertussis or 1μg MBP) almost all 6 month old animals developed disease but not a single 24 month old animal developed EAE under these conditions. They concluded that this indicates a deficit in the recipient and possibly in the non-specific components of the inflammatory response in the CNS. However, none of their rats seemed to be free of disease and extensive leukemic infiltration of lymphoid organs might have immunosuppressed recipients. In the study reported in this chapter, two Lewis (JC) rats which had very extensive abdominal swelling were refractory to EAE and were discarded from the experiment. Due to the array of pathologic lesions found in aged F344 rats (Sass et al., 1975; Levine and Sowinski, 1976), it appears that F344 rats are not a good model for assessing age related factors whereas Lewis (JC) rats, due to being a healthy and more long-lived strain, are a more appropriate model.

Sex steroid hormones have previously been found to influence normal immune mechanisms (Cohn, 1979) and the development of autoimmune disease (Talal et al., 1984). In this study, females generally showed a less severe and less chronic form of the disease. In contrast, generally there is a marked predominance of autoimmune diseases in females and in autoimmune models, androgens have been found to suppress and estrogens to accelerate disease severity (Roubinian et al., 1978; Ahmed and Penhale, 1982). For instance, in murine lupus, a model for systemic lupus erythematosus (SLE), females have a more severe form of the disease (Roubinian et al., 1977). Castrated males develop an accelerated autoimmune disease indistinguishable from females (Roubinian et al., 1978). However, sterilisation of females fails to improve disease severity (Raveche et al., 1979) suggesting that it is androgen which is causing the immunosuppressive effect in this model of autoimmunity. Keith (1978a) reported a sex-related difference in the incidence of spontaneous recurrent EAE. Although the mean onset and the severity of disease was similar in both males and females, 45% of females had a recurrent episode as severe as the initial attack. In contrast, no males exhibited a second episode of disease. However, 15% of males died whereas all females survived both episodes of disease. Hormonal changes due to pregnancy have also been shown to have an immunosuppressive effect on the course of EAE in rats and guinea pigs (Keith, 1978b).

Although the clinical signs of EAE were found to be considerably delayed in the pregnant animals, if disease did occur, it was of a similar severity in pregnant and non-pregnant animals. However, histologically pregnant animals exhibited more severe lesions, suggesting that pregnancy only has a delaying effect on the onset of the disease and does not reduce the severity of disease (Keith, 1978b). In the study described in this chapter, middle-aged sterilised females were compared with non-sterilised females of the same age using both actively and passively induced disease models of EAE to test whether hormonal levels were contributing to the chronicity of disease evident in older animals. The sterilisation of females had no detrimental effect on the onset or severity of clinical EAE in middle-aged animals, however, it may be appropriate to test young sterilised animals as well.

In this study, MBP-specific antibody production did not correlate with onset, severity or relapse of EAE. These are similar results to those found in previous studies where MBP-specific antibody levels were determined in young animals (Lisak et al., 1969; Lennon et al., 1971; Tabira and Endoh, 1985). Anti-MBP antibody levels were found to reach a peak by day 30 post inoculation and when measured at 80 days post inoculation were found to be still at a high level. No significant differences were found between young and aged animals in the production of MBP-specific antibody. Although there is no direct evidence suggesting that antibody plays a role in the pathogenesis of EAE, a role for circulating factors has been suggested in EAE (Bornstein and Appel, 1961; Brosnan et al., 1983). B cell and immunoglobulin-deficient rats fail to develop clinical or histological evidence of EAE when sensitized with whole spinal cord or MBP (Willenborg, 1983). MBP-specific antibody can be detected in serum by day 6 after antigenic challenge (Gonatas et al., 1974) and MBP is found in the antigen depot for at least a year after injection (Tabira et al, 1984). As suggested by some researchers (Paterson and Harwin, 1963; Willenborg, 1979, 1980), it is possible that antibody may play a role in demyelination within inflammatory lesions, and in the recovery and resistance to re-induction of EAE.

Neonatal rats do not show clinical or histopathological evidence of disease after the transfer of EAE effector cells. However, these rats are thought to be asymptomatic carriers of the disease as previous studies (Willenborg, 1986) have shown that if these animals are actively sensitised with MBP in CFA at 10 to 12 weeks of age, they will develop disease significantly earlier than control animals (7 to 8 days compared with 10 to 11 days). Animals which had received effector cells neonatally were found to not only develop disease earlier but MBP antibody levels also increased at Day 5 rather than Day 7. The earlier onset of disease in rats that are neonatally induced is thought to be a memory response due to the persistence of transferred cells (Willenborg et al., 1986). In this study, this memory response was also evident in aged animals 13 months after receiving the effector cells, suggesting that an early etiological event may set up an autoimmune carrier state which can persist even into middle-age. These results support the hypothesis put forward by many researchers that MS is caused

by a viral infection occurring in childhood or adolescence which induces prolonged immunological memory to CNS antigens (Wege et al., 1984; Tardieu et al., 1984; Waksman, 1988). Coupled with the observation in this chapter that EAE is more severe in aged animals, in MS an early etiological event may set up an autoimmune carrier state which can persist even into middle-age and be reactivated with chronic disease resulting due to the aged persons inability to resolve inflammation effectively.

In conclusion, the results presented in this chapter suggest that age-related factors can cause a shift in EAE in Lewis (JC) rats from a self-limiting disease to a chronic or relapsing disease of the CNS. The age-related mechanisms affecting EAE may be complex as age modifies both the immune and endocrine systems. Subsequent chapters will examine in detail factors that may explain the differences in EAE chronicity between young and aged animals.

4.4 Summary

EAE was produced in young (2-3 month old), middle-aged (12-13 month old) and geriatric (24-26 month old) Lewis (JC) rats by active immunisation with MBP in CFA. It was found that aged Lewis (JC) rats develop a more chronic form of EAE than younger rats of the same strain. Active induction shows a slower and less vigorous response in the first instance in aged animals, but the chronicity does not resolve suggesting there is a problem in the resolution of the immune response at the inflammatory site in older animals. In contrast, no significant difference in the production of MBP-specific antibodies was found between young and aged animals. Males exhibited an increased severity and chronicity of disease compared with females of the same age. It was found that at all ages, females had a mean day of onset approximately 2 days after males of the same age, fewer deaths, less numbers of days with paralysis with generally a shorter duration of disease. However, the sterilisation of females had no detrimental effect on the onset or severity of clinical EAE in middle-aged animals. The memory response previously shown in young animals which had received EAE effectors postnatally was also evident in aged animals 13 months after receiving the effector cells. In terms of MS, these results suggest that an early etiological event may set up an autoimmune carrier state which can persist even into middle-age and be reactivated with chronic disease resulting due to the aged persons inability to resolve inflammation effectively.

CHAPTER 5: AGE-RELATED CHANGES IN EAE-INDUCED INFLAMMATION OF THE CNS

5.1 Introduction

As discussed in Chapter 4, the chronicity of actively induced EAE was significantly increased in aged rats (over one year old). The study presented in this chapter aimed to determine whether these clinical differences could be attributed to gross differences in the histopathology of the disease in aged animals.

In EAE, the correlation between the number of inflammatory lesions in the CNS and flaccid paralysis is poor (Hoffman et al., 1973; Raine, 1980; Simmons et al., 1982, 1984). A number of hypotheses have been proposed to explain this apparent paradox. The extent of oedema in the spinal cord has been one of the suggested mechanisms of neurological dysfunction (Levine et al., 1966; Leibowitz and Kennedy, 1972; Simmons et al., 1982; Sedgewick et al., 1987; Butter et al., 1989). The accumulation of inflammatory cells is thought to initiate oedema formation and result in fibrin deposition as vascular permeability allows extravasation of plasma proteins into the perivascular space where fibrinogen is converted to insoluble fibrin and deposited (Colvin and Dvorak, 1975). Fibrin is deposited in lesions of EAE (Oldstone and Dixon, 1968; Paterson, 1976; Rauch et al., 1978) and in histological sections of spinal cord from Lewis rats with acute EAE, Ackerman et al. (1981) found that fibrinogen was restricted to vessels containing cellular infiltrates. These results suggested that fibrin deposition does not occur as a result of general vascular permeability, but is produced by infiltrating cells. As fibrinogen is a large molecule (340,000 daltons) it can possibly only enter lesions when vascular damage has occurred. Neural cells are also a rich source of thromboplastin and any damage in the CNS can lead to the release of thromboplastin and additional fibrin formation (Graebar and Stuart, 1978). These results suggest that oedema associated with fibrin deposition may cause a loss of conduction of impulses down nerve fibres which results in paralysis in EAE. The subsequent resolution of oedema may be the initial factor contributing to the recovery from disease.

In contrast, it has been suggested by others that it is demyelination which produces the clinical signs of EAE (Pender, 1987; Pender et al., 1990). The insulation of axons with myelin is essential for the high-speed conduction of action potentials as the myelin sheath shields against the capacitance and conductance of the internode (Morrell and Norton, 1980). In myelinated axons, the action potential propagates discontinuously, in saltatory jumps from one node of Ranvier to the next, which is a faster process than the continuous propagation of unmyelinated axons (Rowland, 1985). Demyelination causes conduction block with refractory periods of transmission (McDonald and Sears, 1970;

Smith et al., 1979,1981; Bostock and Grafe, 1985; Kaji et al., 1988). Motor conduction abnormalities have been found in the region of the spinal cord ventral roots in acute EAE in Lewis rats (Pender, 1988b). However, the clinical form and amount of demyelination are different depending upon the species and antigens used. Chronic relapsing EAE, like MS, is characterised by extensive demyelination associated with lesions. Guinea pigs show the greatest amount of demyelination and the least axonal damage with more demyelination seen in animals with a more chronic disease process (Tabira and Sakai, 1987). Guy et al. (1991) measured myelin sheath thickness and axon diameter of optic nerves in guinea pigs suffering from EAE. Both myelin sheath thickness and axonal diameter of animals with EAE were significantly lower than normal animals. Jones et al. (1990) found that prominant perivascular demyelination associated with mononuclear inflammation was evident in passively induced EAE in Buffalo rats and that demyelination occurred without the addition of demyelinating antibodies or subsequent clinical relapses. In contrast, there are numerous claims that demyelination cannot be the cause of neurological signs of acute EAE as demyelination is reported to be sparse or absent in these models, particularly if MBP is the inoculum (Lampert, 1965; Hoffman et al., 1973; Lassman and Wisniewski, 1979; Panitch and Ciccone, 1981; Raine et al., 1981; Simmons et al., 1981, 1983; White, 1984; Kerlero de Rosbo et al., 1985). Although MBP is necessary for the induction of the inflammatory response, demyelination may be caused by an additional immune response against other lipid myelin surface antigens (Moore et al., 1984). In addition, demyelination occurs in cerebellar cultures if EAE sera from animals immunised with whole CNS tissue is used (Bornstein and Appel, 1961) but not if MBP is used as the immunogen (Seil et al., 1968).

Remyelination is also evident in EAE (Lampert, 1965; Wisniewski et al., 1969; Lassman et al., 1980b) and has been proposed to play a role in disease recovery although it has generally been considered to be a late feature of demyelinated chronic lesions (Prineas et al., 1969). As remyelination occurs, conduction is restored resulting in a return to transmission (Smith et al., 1979, 1981). Koles and Rasminsky (1972) found that as little as 3% of normal myelin thickness could be sufficient to ensure saltatory conduction. Pender (1988) performed histological and electrophysiological studies and found that the functional recovery from acute EAE in Lewis rats was due to the remyelination achieved by Schwann cells in the PNS and oligodendrocytes in the CNS. Raine and Traugott (1983) found that animals treated with a combination of MBP and galactocerebroside during chronic relapsing EAE displayed widespread remyelination and oligodendroglial proliferation. Others propose that disease recovery occurs too rapidly to be attributed to remyelination (McDonald, 1974a,b; Panitch and Ciccone, 1981; Simmons et al., 1981).

Finally, astrocytes which form scar tissue in response to injury to the nervous system (reactive gliosis) (Miller et al., 1986) may also be involved in the pathogenesis of EAE. Scars are formed by astrocytes extending numerous processes that become larger and have a substantial increase in glial filaments (Maxwell and Kruger, 1965; Eng, 1985). The proliferating astrocytes engage in phagocytosis (Noske et al., 1982; Kandel, 1985; Eng, 1985), and consequently astrocytes remove debris and help seal-off damaged brain tissue after neuronal death or injury (Kandel, 1985). This encroachment of astrocytes displaces presynaptic terminals and results in the damaged neurons receiving reduced synaptic input (Kandel, 1985). Fibrillary astrogliosis is one of the preclinical changes evident in EAE (Field, 1961; Bubis and Luse, 1964; Lampert, 1967). Gliosis is a prominant feature in the chronic model of EAE in the guinea pig and mouse (Linington et al., 1984; Raine, 1983; Smith et al., 1984, 1985). Smith et al. (1983) have shown that in acute EAE in the Lewis rat enhanced immunocytochemical staining of GFAP is evident 10 to 12 days post inoculation. This intensity of staining increased with time post inoculation. Reactive astrocytes were distributed throughout the tissue and persisted until clinically evident disease had subsided, with the increased staining correlating with an increased permeability of the BBB. An important observation in the study presented in Chapter 3 was that astrocytic proliferation was evident in irradiated and non-irradiated animals exhibiting advanced clinical signs of EAE. These results suggest that astrocytes may play an important role in the pathogenesis of EAE.

The specific aims of the study presented in this chapter were to establish whether there were age-related differences in histopathological features of EAE such as lesion formation, fibrin deposition, demyelination and astrocytic hypertrophy.

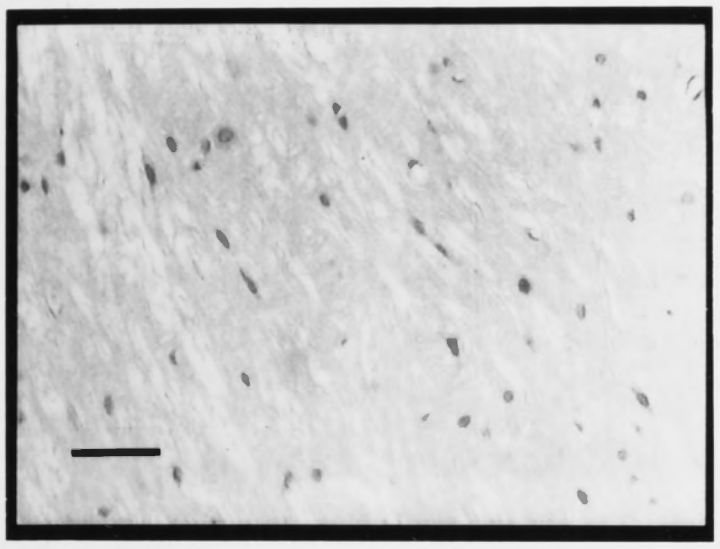
5.2 Results

5.2.1 Accumulation of inflammatory cells in the spinal cord

As EAE histologically presents with the accumulation of inflammatory cells in the CNS, the question whether lesion formation differed in young and middle-aged animals during the development of the disease was addressed. Inflammatory lesions became apparent in young and middle-aged animals during the onset of disease (Fig. 5.1). Histological examination revealed meningeal and subpial infiltrates and perivascular cuffs and infiltrates of mononuclear cells evident in the spinal cord by day 14 post inoculation in both young (Fig. 5.1b) and middle-aged (Fig. 5.1f) animals. Inflammatory lesions were not evident in naive animals (Fig. 5.1a,e). Inflammatory lesions were considerably resolved by day 22 post inoculation in middle-aged animals (Fig. 5.1g) whereas young animals (Fig. 5.1c) still exhibited considerable inflammatory infiltrates. By day 40 post inoculation, there was little evidence of inflammatory infiltrates in young or middle-aged animals (Fig. 5.1d,h). Quantification of histological sections verified the impression

Fig. 5.1: Hematoxylin and eosin stained histological sections of parenchymal regions of spinal cord from male rats inoculated with 50μg MBP in CFA containing 400μg *M. butyricum*. Representative photographs of the presence or absence of inflammatory lesions found in young animals at days 0 (a), 14 (b), 22 (c) and 40 (d) and middle-aged animals at days 0 (e), 14 (f), 22 (g) and 40 (h). Sections were viewed using video imaging at x225 (scale bar corresponds to 50μms).

a)



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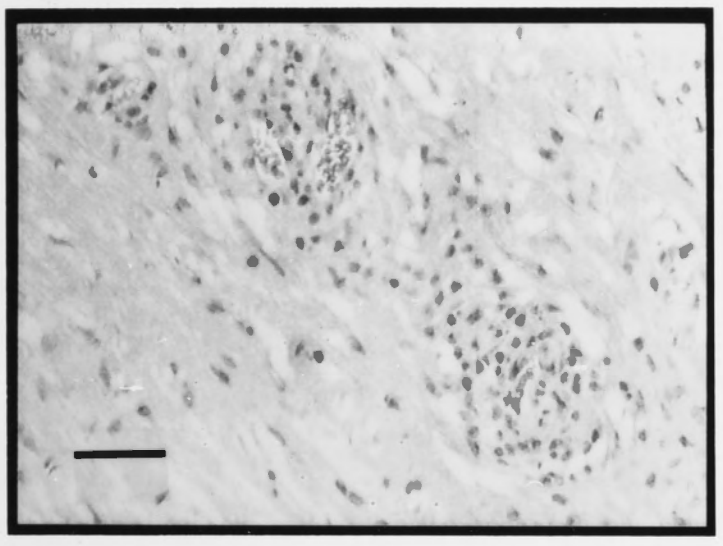

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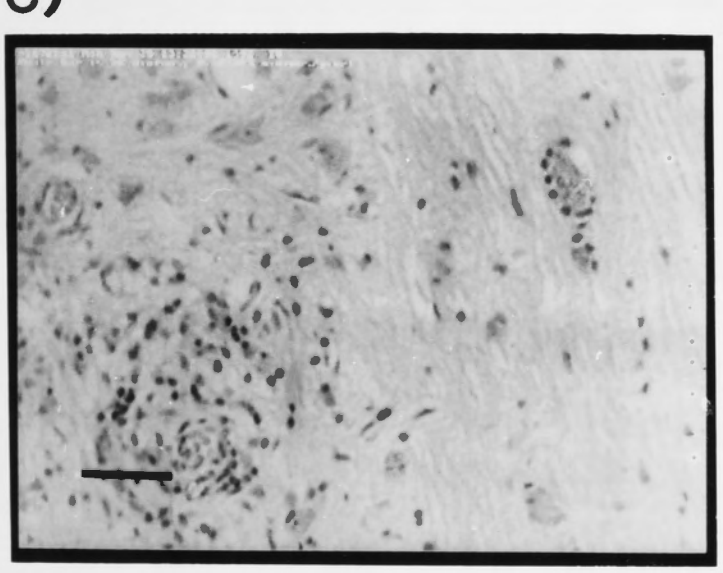

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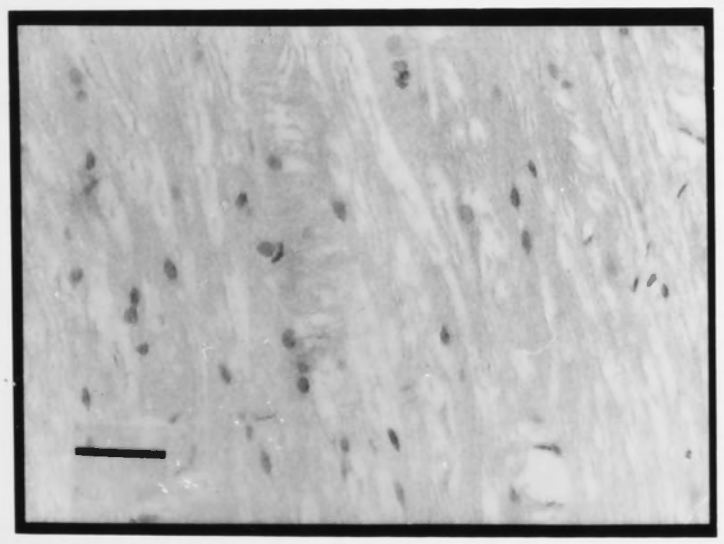
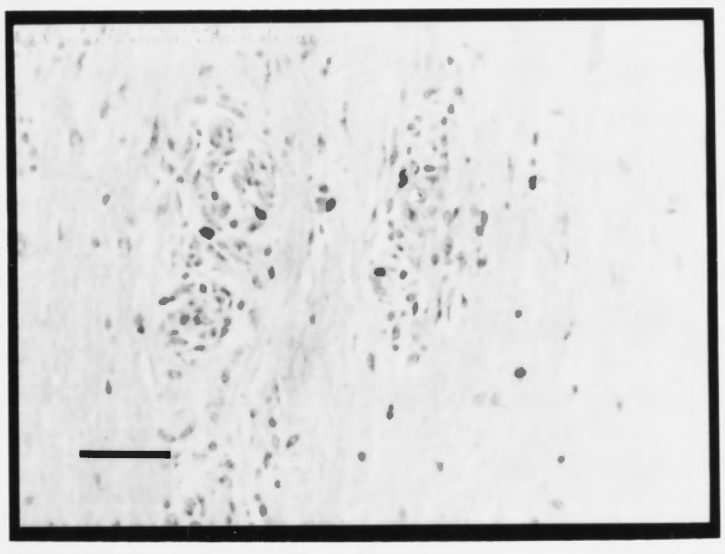
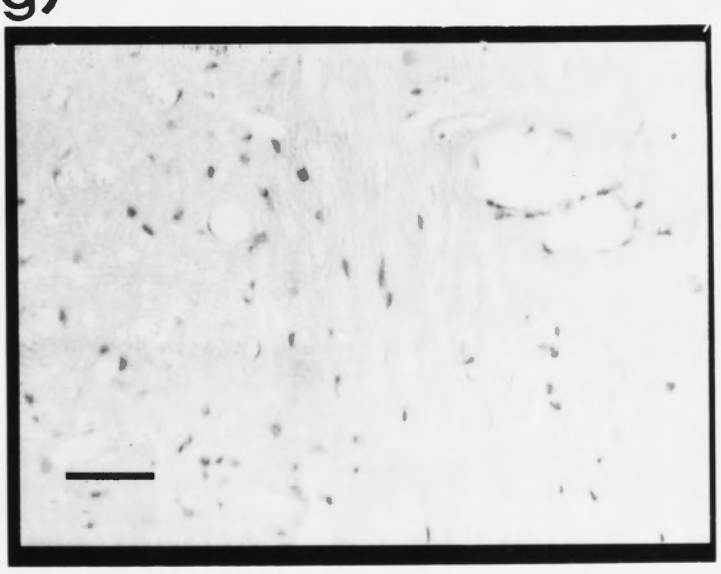


e)

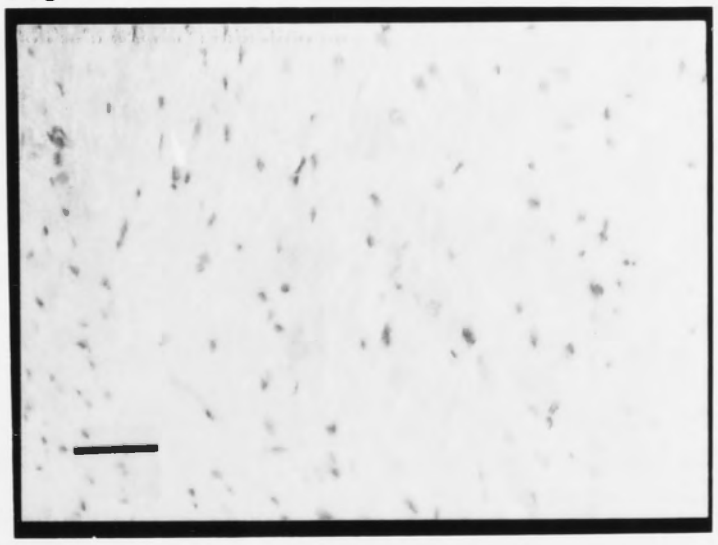
f)



g)



h)



gained from microscopic examination (Table 5.1). Lesions were virtually absent in animals at day 7, prior to the onset of disease but were numerous in all regions of the spinal cord by day 14 in young and middle-aged animals, which correlated with an increase in disease severity (clinical scores of 4.6 ± 0.2 and 3.9 ± 0.6 respectively). However, despite the absence of clinical signs in young animals at day 22 post inoculation, these animals had a considerable number of lesions (eg., 19.4 ± 0.5 lesions in the lumbar spinal region), whereas middle-aged animals despite a mean clinical score of 2.5 ± 0.5 had few lesions (eg., 0.7 ± 0.3 lesions in the lumbar spinal cord). By day 40 post inoculation, inflammatory lesions were almost totally resolved in both young and middle-aged animals, despite the presence of clinical signs in 29% of middle-aged animals. These results suggest that although the peak of disease correlated with a substantial number of inflammatory lesions, the chronicity of disease in middle-aged animals was not due to the persistence of large numbers of inflammatory lesions in the spinal cord.

5.2.2 Fibrin deposition

To assess whether the significant increase in severity and chronicity of disease in middle-aged animals was due to increased fibrin deposition, lumbar spinal cord regions were sectioned and stained with MSB fibrin and examined by light microscopy. It was evident that fibrin deposition was not significantly increased above background during the course of disease. Representative photographs of the presence of fibrin are shown in young (Fig. 5.2a,b,c,d) and middle-aged (Fig. 5.2e,f,g,h) animals. As an additional method for the detection of fibrin deposition, electron microscopy was performed on lumbar spinal cord sections (data not shown) and again no increase in fibrin deposition above background were observed, suggesting that fibrin deposition does not play a major role in the clinical signs of disease in this model of EAE.

5.2.3 Demyelination

To assess whether the significant increase in severity and chronicity of disease in middle-aged animals was due to increased demyelination, lumbar spinal cord regions were sectioned and stained with chromoxane cyanine R and examined by light microscopy. Demyelination was not evident during the course of disease. Representative photographs of myelinated areas are shown in young (Fig. 5.3a,b,c,d) and middle-aged (Fig. 5.3e,f,g,h) animals. These results suggest that demyelination does not cause the clinical signs of disease in this model of EAE.

TABLE 5.1

NUMBER OF INFLAMMATORY LESIONS IN SPINAL CORD SECTIONS OF YOUNG AND MIDDLE-AGED MALE RECIPIENTS

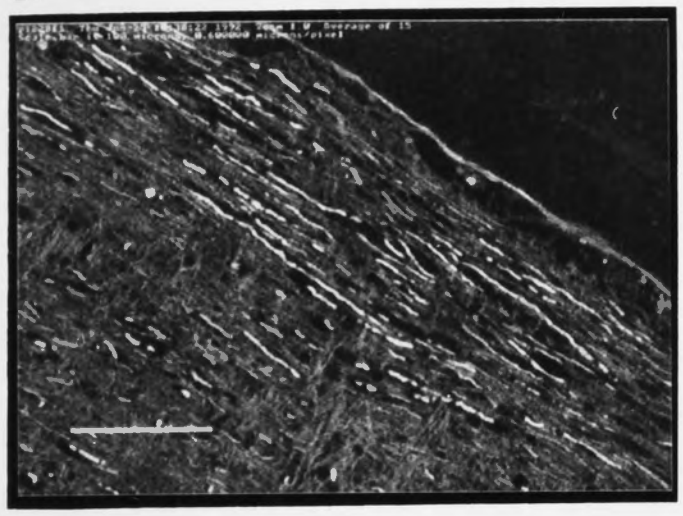
D.P.I.[a]	YOUNG					MIDDLE-AGED				
	N	Clinical Score	No. of lesions			N	Clinical Score	No. of lesions		
			Lumbar	Thoracic	Cervical			Lumbar	Thoracic	Cervical
0	4	0	0	0	0	4	0	0	0	0
7	2	0	0.4 ± 0.1[b]	0.2 ± 0.1	0.1 ± 0.1	2	0	0.2 ± 0.1	0	0.1 ± 0.1
14	7	4.6 ± 0.2	54.8 ± 3.1	48.4 ± 3.5	50.9 ± 3.9	7	3.9 ± 0.6	47.3 ± 3.0	38.0 ± 5.9	30.6 ± 4.7
22	2	0	19.4 ± 0.5	15.5 ± 3.7	18.2 ± 1.0	2	2.5 ± 0.5	0.7 ± 0.3	1.5 ± 0.5	1.3 ± 0.9
40	4	0	0.3 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	7	0.8 ± 0.5	1.4 ± 0.6	2.7 ± 1.5	0.6 ± 0.3

[a] Days post inoculation

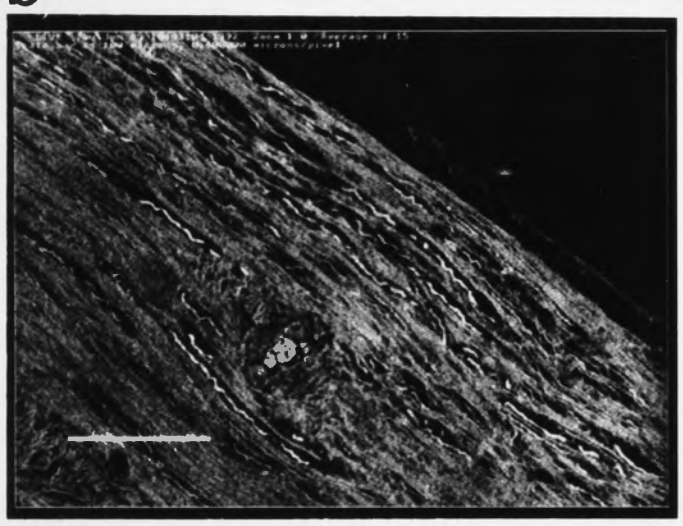
[b] ± se

Fig. 5.2: MSB fibrin stained histological sections of parenchymal regions of spinal cord from male rats inoculated with 50μg MBP in CFA containing 400μg *M. butyricum*. Representative photographs of the presence or absence of fibrin deposition found in young animals at days 0 (a), 14 (b), 22 (c) and 40 (d) and middle-aged animals at days 0 (e), 14 (f), 22 (g) and 40 (h). Sections were viewed using confocal fluorescence imaging at x200 (scale bar corresponds to 100μms). Fluorescent areas represent fibrin deposition.

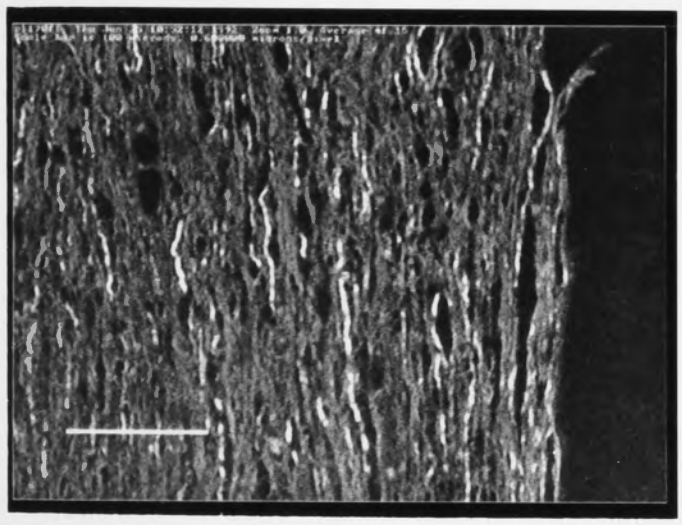
a



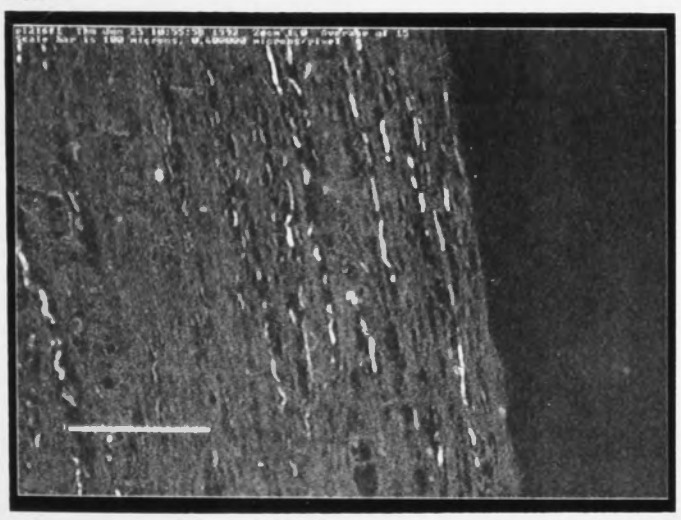
b



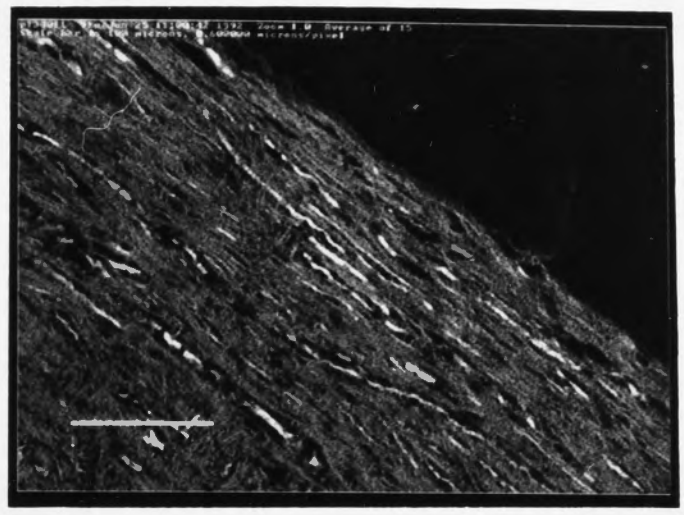
c



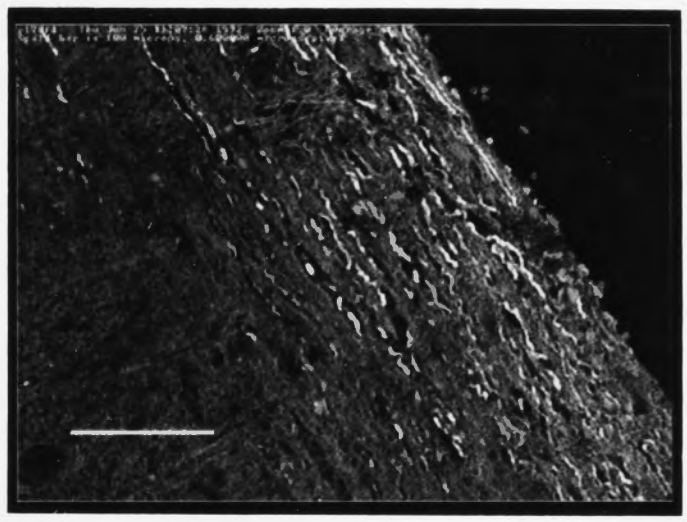
d



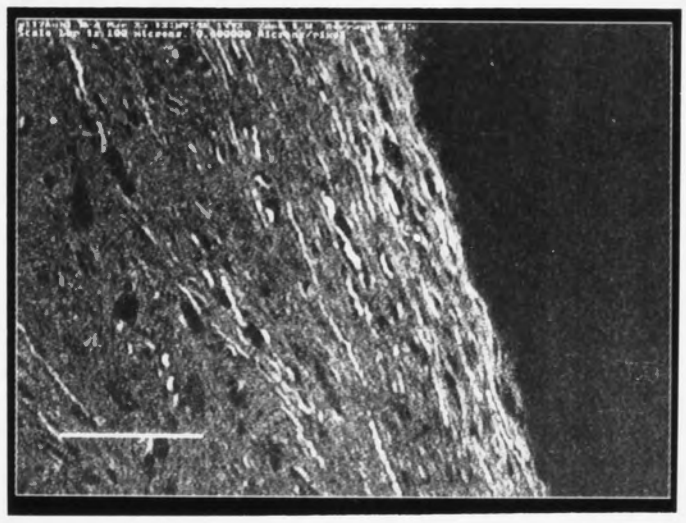
e



f



g



h

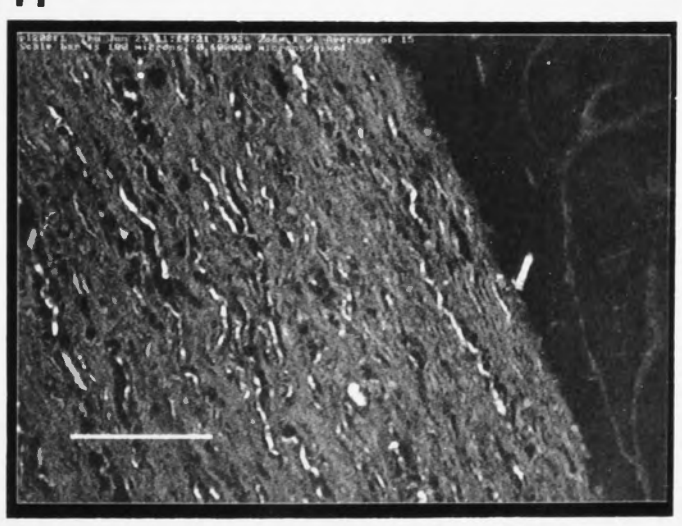
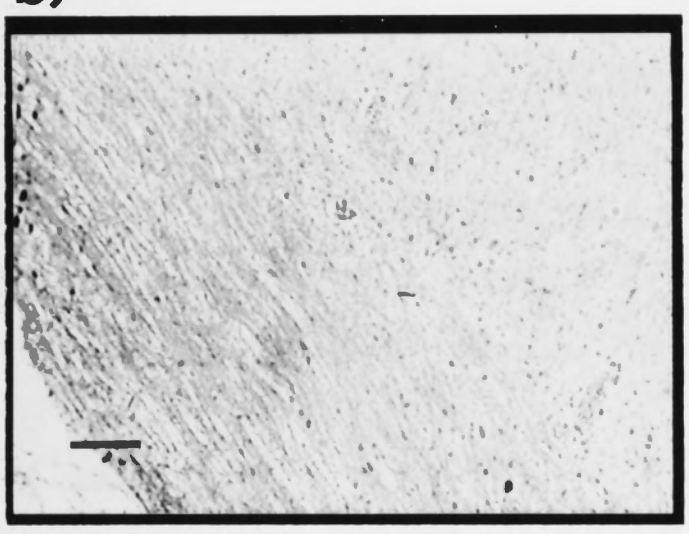


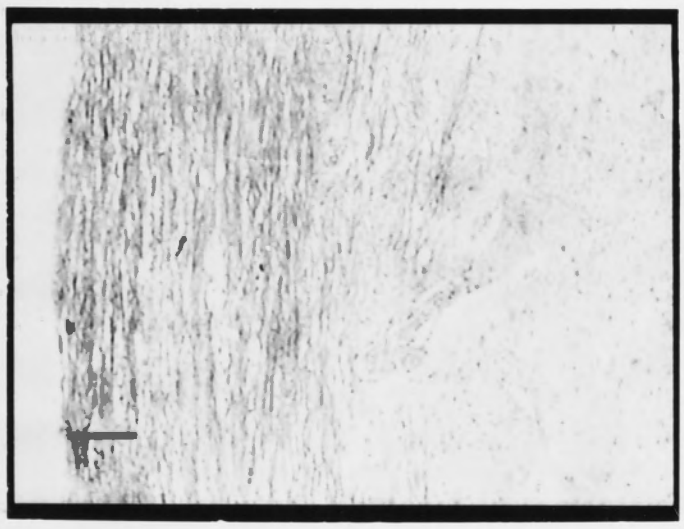
Fig. 5.3: Chromoxane cyanine R (stain for myelin) stained histological sections of parenchymal regions of spinal cord from male rats inoculated with 50μg MBP in CFA containing 400μg *M. butyricum*. Representative photographs of myelinated areas found in young animals at days 0 (a), 14 (b), 22 (c) and 40 (d) and middle-aged animals at days 0 (e), 14 (f), 22 (g) and 40 (h). Sections were viewed using video imaging at x90 (scale bar corresponds to 100μms).

a)

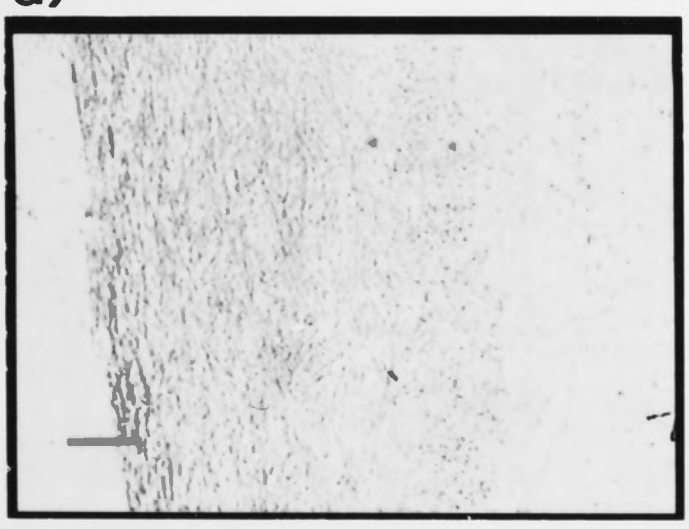
b)



c)

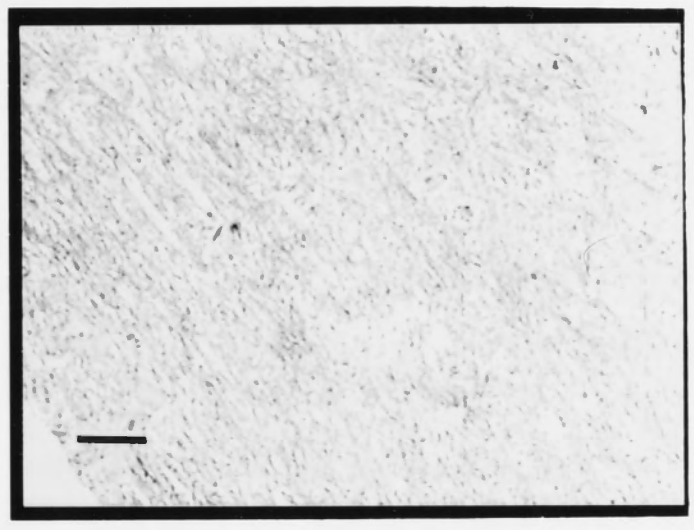


d)



e)

f)



g)

h)

5.2.4 Astrocytic hypertrophy

The hypertrophy of astrocytes, which consists of the extension of numerous processes that become larger and have a substantial increase in glial filaments, is the astrocytic response to CNS damage (Maxwell and Kruger, 1965; Eng, 1985). To assess whether the significant increase in chronicity of disease in middle-aged animals was due to increased astrocytic hypertrophy, lumbar spinal cord regions were sectioned and stained with mercuric chloride and examined by light microscopy. By using this modification of Cajal's (1913) method (Ralis et al., 1973), it was found that reactive astrocytes and their processes stained intensely, while resting astrocytes were inconspicuous. It was evident that the presence of reactive astrocytes increased during the course of disease (Table 5.2). Reactive astrocytes were absent in naive animals, however by day 14 post inoculation were found distributed throughout the tissue in young (1.9 ± 0.3) and middle-aged (1.6 ± 0.2) animals. In Fig. 5.4, representative photographs of the presence of astrocytic hypertrophy are shown in young (Fig. 5.4a,b,c,d) and middle-aged (Fig. 5.4e,f,g,h) animals. At day 22 post inoculation, although reactive astrocytes were still evident in young animals (1.7 ± 0.3), these astrocytes were faintly staining and only 14.6% had long processes (Fig. 5.4c). In contrast, reactive astrocytes were clearly evident in middle-aged animals (3.0 ± 0.0) with 77% of reactive astrocytes with long intensely staining astrocytic processes (Fig. 5.4g). At day 40 post inoculation, the presence of reactive astrocytes was considerably resolved in young animals (0.4 ± 0.2) with only 1.1% of these astrocytes exhibiting evidence of hypertrophy. In contrast, reactive astrocytes were evident in middle-aged animals (2.1 ± 0.3) with 42% with long processes (Fig. 5.4h). In addition, at 40 days post inoculation, sections from middle-aged animals exhibiting signs of EAE showed the presence of intensely staining astrocytes (2.9 ± 0.1) with $74\% \pm 9.0$ with long processes, whereas in asymptomatic animals although reactive astrocytes were still present (1.6 ± 0.3), there were significantly fewer and these were less intensely stained with shorter processes (only $13.0\% \pm 7.9$ had long processes). Representative photographs of an asymptomatic (Fig. 5.5a) and an animal exhibiting clinical signs of EAE (Fig. 5.5b) show the considerable difference found in the degree of astrocytic hypertrophy. These results suggest that astrocytes play a major role in the chronicity of disease shown in middle-aged animals in this model of EAE.

5.3 Discussion

In Chapter 4, middle-aged rats were found to develop a more chronic form of EAE than younger rats of the same strain. In order to determine whether the clinical differences could be attributed to gross differences in the histopathology of the disease in middle-aged animals, the inflammatory lesions were quantified, and the degree of fibrin

TABLE 5.2

PRESENCE OF ASTROCYTIC HYPERTROPHY IN SPINAL CORD SECTIONS OF YOUNG AND MIDDLE-AGED MALE RECIPIENTS

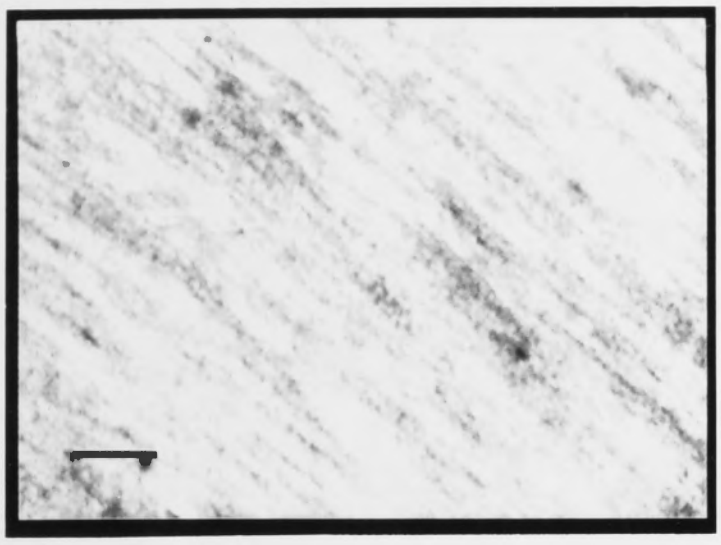
	YOUNG				MIDDLE-AGED			
D.P.I.[a]	N	Clinical Score	Presence of activated astrocytes[b]	Percentage with long processes	N	Clinical Score	Presence of activated astrocytes	Percentage with long processes
0	4	0	0	0	4	0	0	0
14	7	4.6 ± 0.2	1.9 ± 0.3	27.6 ± 10.1	7	3.9 ± 0.6	1.6 ± 0.2	32.0 ± 7.0
22	2	0	1.7 ± 0.3	14.6 ± 5.7	2	2.5 ± 0.5	3.0 ± 0.0	77.0 ± 13.9
40	4	0	0.4 ± 0.2	1.1 ± 0.7	5	1.1 ± 0.6	2.1 ± 0.3	42.0 ± 16.6

[a] Days post inoculation

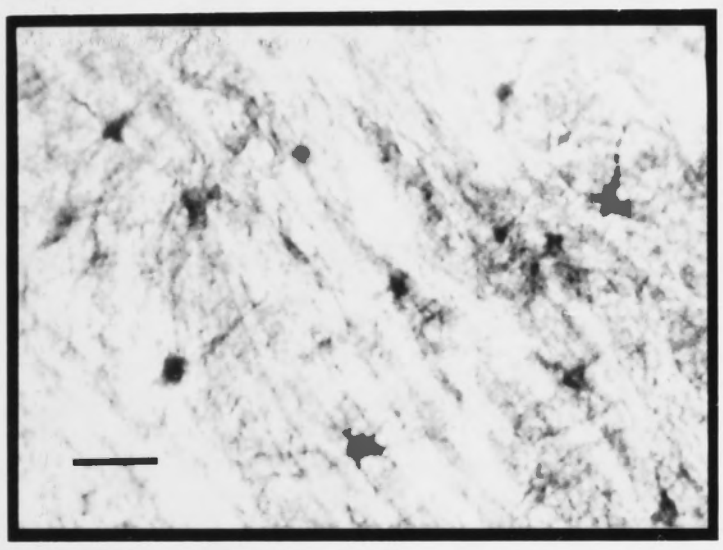
[b] The presence of astrocytes in the spinal cord sections were scored on a scale from 0 to 3, where 0 = no astrocytes; 1 = <5 astrocytes per field; 2 = 5 to 9 astrocytes per field; 3 = 10 or more astrocytes per field

Fig. 5.4: Mercuric chloride stained astrocytes in histological sections of parenchymal regions of spinal cord from male rats inoculated with 50μg MBP in CFA containing 400μg *M. butyricum.* Representative photographs of the presence or absence of reactive astrocytes found in young animals at days 0 (a), 14 (b), 22 (c) and 40 (d) and middle-aged animals at days 0 (e), 14 (f), 22 (g) and 40 (h). Sections were viewed using video imaging at x225 (scale bar corresponds to 50μms).

a)



b)



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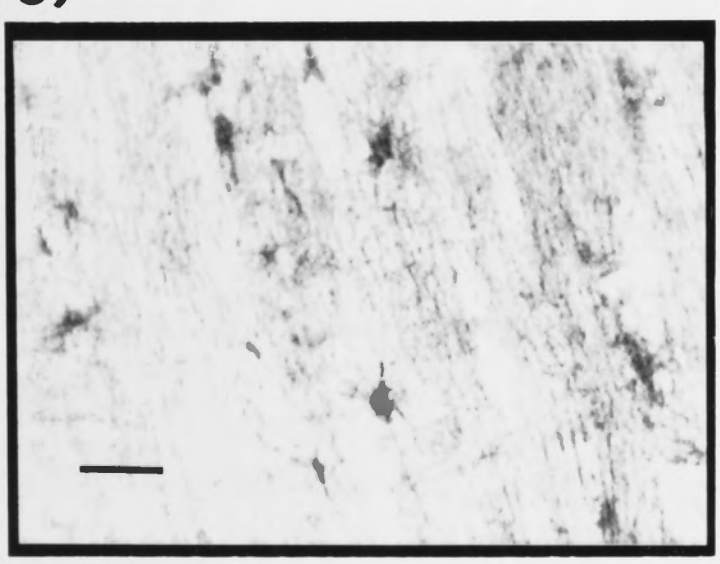
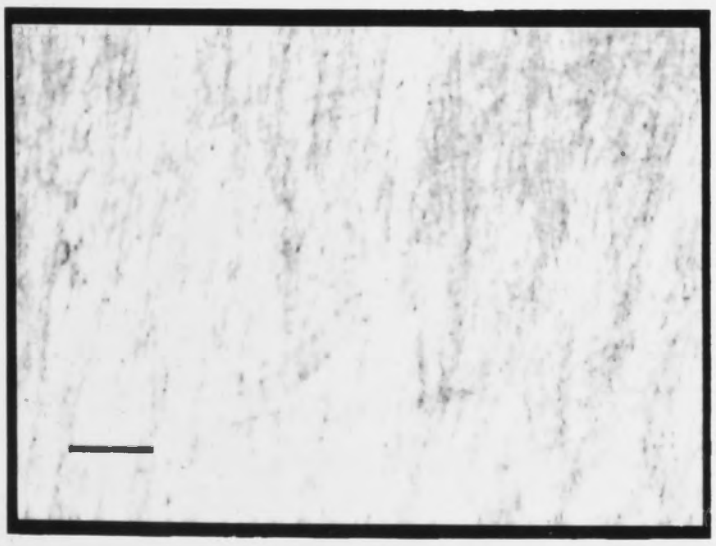
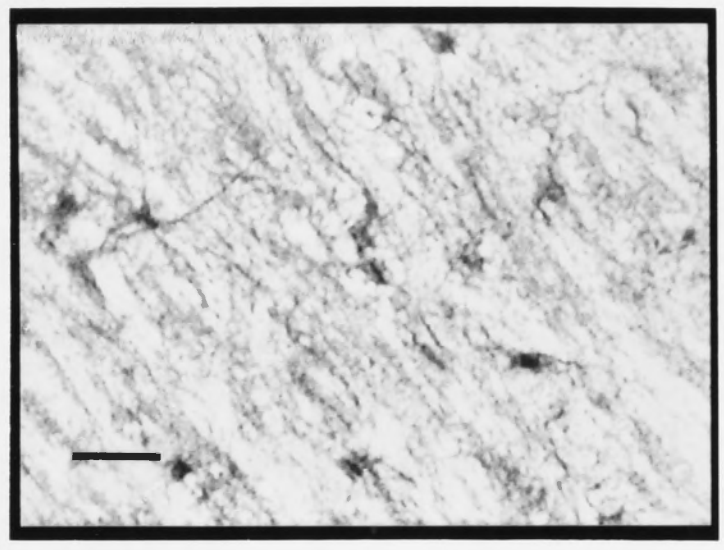


d)

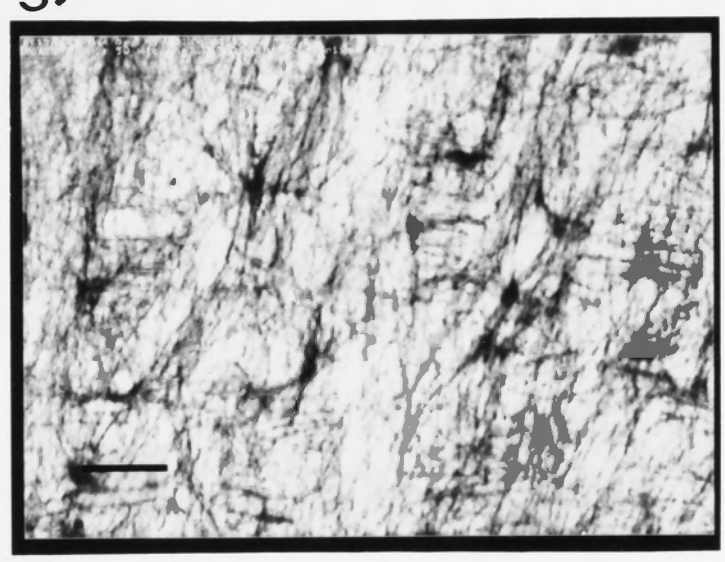


e)

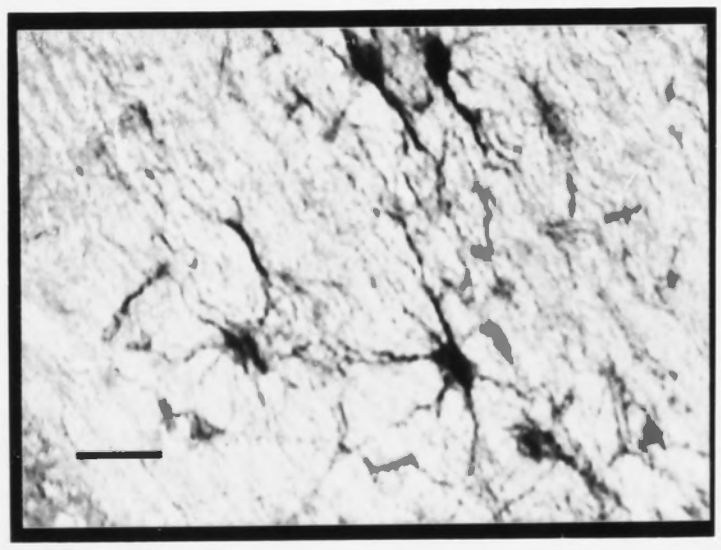
f)



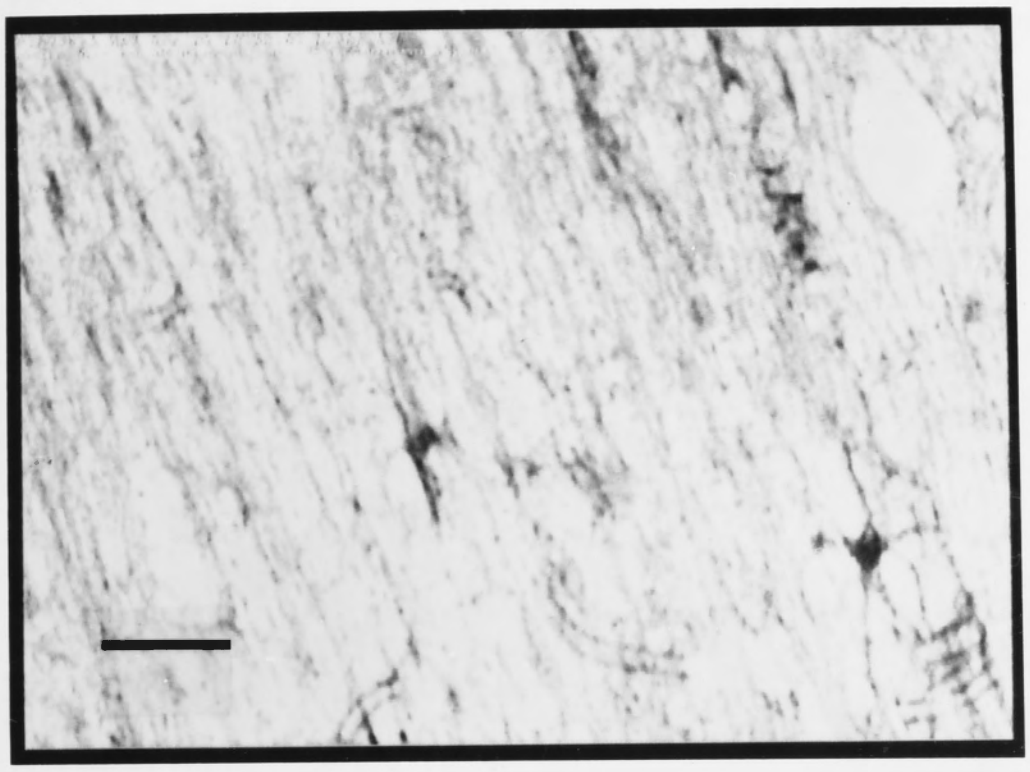
g)



h)



a)



b)

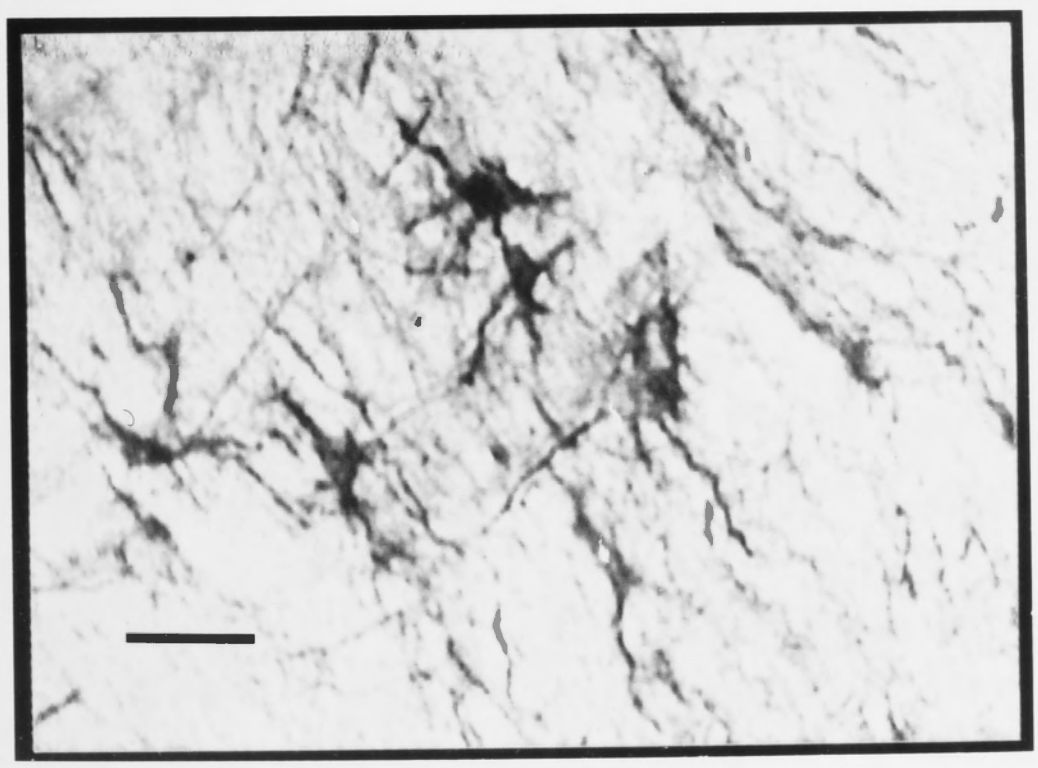


Fig. 5.5: Mercuric chloride stained astrocytes in histological sections of parenchymal regions of spinal cord from middle-aged male rats inoculated with 50µg MBP in CFA containing 400µg *M. butyricum.* Representative photographs of the presence of reactive astrocytes found in an asymptomatic animal (a) and an animal exhibiting clinical signs of EAE (b) at day 40 post inoculation. Sections were viewed using video imaging at x225 (scale bar corresponds to 50µms).

deposition, demyelination and astrocytic hypertrophy was compared. Inflammatory lesions became apparent before the onset of disease, increased significantly during advanced clinical signs of EAE and were still evident at day 22 in young animals despite the absence of clinical signs but were largely absent from middle-aged animals which still exhibited disease symptoms. At day 40 post inoculation inflammatory infiltrates were sparse in both young and middle-aged animals despite 29% of middle-aged animals being symptomatic. These results suggest that disease chronicity does not correlate with the number of inflammatory lesions. No significant differences were found in the degree of fibrin deposition or demyelination between young and middle-aged or symptomatic and asymptomatic animals. However, astrocytic hypertrophy was found to correlate with disease chronicity, suggesting that astrocytic hypertrophy may play a significant role in the pathophysiology of the disease.

It has previously been found in EAE, that the correlation between the number of lesions in the CNS and flaccid paralysis is poor (Hoffman et al., 1973; Raine, 1980; Simmons et al., 1982, 1984). Similarly, in the study presented in this chapter, it was evident that the persistence of inflammatory lesions found in young animals at day 22 did not cause chronicity of disease. In addition, the resolution of lesions apparent in middle-aged animals at days 22 and 40 post inoculation suggest that the persistence of the neurological deficit found in middle-aged animals is not due to inflammatory lesions.

Although fibrin has previously been found to be deposited in lesions of EAE (Oldstone and Dixon, 1968; Paterson, 1976) and has been suggested as a cause of paralysis in EAE (Paterson, 1976), no significant fibrin deposition above background was found in this model of EAE in young and middle-aged or symptomatic and asymptomatic animals using both light and electron microscopic methods for the detection of fibrin. The reason for this discrepancy with earlier studies is unclear. However the results clearly indicate that fibrin deposition does not cause the chronicity of the neurological deficit found in middle-aged animals using this particular model of EAE.

In this model of EAE, demyelination was not apparent in young and middle-aged, or symptomatic and asymptomatic animals. Demyelination has previously been reported to be sparse or absent in acute models of EAE, particularly if MBP is the inoculum (Hoffman et al., 1973; Lassman and Wisniewski, 1979; Panitch and Ciccone, 1981; Raine et al., 1981; Simmons et al., 1981, 1983; White, 1984; Kerlero de Rosbo et al., 1985). It has also been suggested that recovery occurs too rapidly to be attributed to remyelination (McDonald, 1974a,b; Panitch and Ciccone, 1981; Simmons et al., 1981). More demyelination has usually been found in animals with a more chronic disease process (Tabira and Sakai, 1987) and it was hypothesised that the more chronic form of disease in middle-aged animals may be due to demyelination. However, this was clearly not the case. The results presented in this chapter suggest that demyelination is not the cause of the persistent neurological deficit found in middle-aged animals in this model of

EAE, although electron microscopic studies would be necessary to discount demyelination evident in the spinal cord ventral roots as suggested by Pender (1988b).

In this study astrocytic hypertrophy was found to increase during the development of disease and persist in symptomatic middle-aged animals. Astrocytic proliferation was also found in irradiated and non-irradiated animals exhibiting advanced clinical signs of EAE (refer to Chapter 3). These results suggest that astrocytes may play an important role in the pathogenesis of EAE. Fibrillary astrogliosis has previously been found to be one of the preclinical changes evident in EAE (Field, 1961; Bubis and Luse, 1964; Lampert, 1967). Gliosis is also a prominant feature in the chronic model of EAE in the guinea pig and mouse (Linington et al., 1984; Raine, 1983; Smith et al., 1984, 1985). In the study presented in this chapter, the intensity of staining increased with time post inoculation with reactive astrocytes being distributed throughout the tissue and decreasing in the intensity of staining as clinically evident disease subsided. Similarly, other investigators have also shown that in acute EAE in the Lewis rat enhanced immunocytochemical staining of GFAP is evident 10 to 12 days post inoculation with staining for GFAP increasing with time (Smith et al., 1983; Goldmuntz et al., 1986; Cammer et al., 1990). Clinical and histological EAE can also be induced in guinea pigs and monkeys by the injection of human glioblastoma, further suggesting that glial cells are associated with the symptoms of EAE (Bigner et al., 1981).

Astrocytes are found in "white and grey matter" areas of the CNS where they are associated with myelinated regions, nerve cell bodies, dendrites and synapses, which they characteristically envelop (Ffrench-Constant and Raff, 1986). It has been suggested that astrocytic gliosis may contribute to the clinical symptoms of EAE if demyelination is a major cause of disease symptoms. In a study on CREAE in the SJL/J mouse, Smith et al. (1988) found astrocytic hypertrophy near demyelinating lesions which eventually resulted in the formation of gliotic plaques in the affected mouse. Astrocytes contain lysosomal enzymes which are activated in EAE lesions and are capable of degrading myelin (Arstila et al., 1973; Allen, 1983). Demyelinated axons are associated with astrocytic processes (Black et al., 1987) and gliosis has been shown to inhibit remyelination *in vitro* (Raine and Bornstein, 1970) and impede regenerating axons in the dorsal roots of the spinal cord (Liuzzi and Lasek, 1987). However, although astrocytic hypertrophy was evident in the study presented in this chapter, demyelination was not and other mechanisms of dysfunction are suggested.

It is known that CD4+ cells are cytotoxic to MBP-presenting astrocytes *in vitro* (Sun and Wekerle, 1986) and possibly CD4+ cells in inflammatory lesions cause damage to astrocytes within the CNS, resulting in the formation of astrocytic hypertrophy and consequently in nerve conduction defects and clinical symptoms of EAE. Kandel (1985) has found that encroachment of proliferating astrocytes can displace presynaptic terminals and result in the damaged neurons receiving reduced synaptic input, with the evoked

excitatory presynaptic potentials being smaller in amplitude. The proliferation of fibrous astrocytes leads to the formation of a glial scar which can subsequently block the restoration of severed synaptic connections (Kelly, 1985). It is possible that the astrocytic hypertrophy evident in middle-aged animals results in chronicity of disease by the long astrocytic processes interfering with synaptic input.

Astrocytes that surround the synaptic region, also have a high affinity for certain neurotransmitters such as gamma aminobutyric acid and serotonin (Bowman and Kimelberg, 1984; Kandel and Schwartz, 1985; Kimelberg and Katz, 1985). Serotonin is thought to play a role in the induction phase of EAE disease as methysergide, a serotonin antagonist, blocks the effect of the pertussigen (Linthicum et al., 1982). The addition of pertussigen in adjuvant results in the induction of EAE in genetically resistant strains of mice (Levine and Sowinski, 1973; Bernard and Carnegie, 1975; Raine et al., 1980a; Munoz and McKay, 1984). Neurological signs of EAE have been attributed to impairment of monoaminergic neurotransmission (Carnegie, 1971; White, 1984). During the acute paralytic phase of EAE in rats, large numbers of the bulbospinal axons that contain the monoamine neurotransmitters, serotonin and noradrenalin appear to be damaged (White et al., 1985). Interference with neurotransmission due to decreases in noradrenaline and serotonin in the lumbar-sacral region (Lycke and Roos, 1973; Honeggar and Isler, 1984), an area rich in serotonin terminals, may account for the localisation of clinical symptoms of EAE (Lennon and Carnegie, 1971). Possibly, astrocytes, by their ability to bind and internalise neurotransmitters, may reduce local concentrations of specific neurotransmitters and consequently interfere with the conduction of impulses.

Astrocytic hypertrophy may be a reaction to the degeneration of neighbouring synapses, neurites or entire neurons (Adams and Jones, 1985; Geinisman et al., 1978; Landfield et al., 1977). As most neurones in the adult mammalian CNS are postmitotic, they will not regenerate after physical injury as would happen elsewhere in the body and functional recovery will depend on the site of injury. It is postulated that neurones require a certain amount of stimulation to survive (Kelly, 1985). Thus, repair mechanisms activated in the CNS by neuronal injury may be detrimental and lead to blocked regeneration of neurones (Kelly, 1985). Consequently, the motor conduction abnormalities previously found in the region of the spinal cord ventral roots in acute EAE in Lewis rats (Pender, 1988b) may be due to astrocytes slowing the conduction of fibers and resulting in clinical signs of EAE. These changes would limit recovery and possibly contribute to neurological deficit.

The immunoregulatory events determining the induction of disease and the subsequent recovery from EAE have not been clearly defined. From the results presented in this study, it seems most likely that astrocytic hypertrophy causes the chronic neurological deficit of EAE seen in middle-aged animals and that the resolution of

astrocytic hypertrophy is one of the factors contributing to the recovery of disease in this particular model of EAE. Possible mechanisms of dysfunction can be attributed to the encroachment of proliferating astrocytes displacing presynaptic terminals which results in the damaged neurons receiving reduced synaptic input; the formation of astrocytic scar tissue blocking the restoration of severed synaptic connections and by their ability to bind and internalise neurotransmitters, astrocytes may reduce local concentrations of specific neurotransmitters and consequently interfere with the conduction of impulses. MS is also a disease in which intense fibrous astrogliosis is a prominant feature (Raine, 1983, 1984) and it is possible that astrocytes contribute to tissue damage by acting as either auxillary or effector cells in this human disease and its animal model EAE.

5.4 Summary

In Chapter 4, middle-aged rats were found to develop a more chronic form of EAE than younger rats of the same strain. In order to determine whether the clinical differences could be attributed to gross differences in the histopathology of the disease in middle-aged animals, the inflammatory lesions were quantified, and the degree of fibrin deposition, demyelination and astrocytic hypertrophy was compared. Inflammatory lesions became apparent before the onset of disease, increased significantly during advanced clinical signs of EAE and were still evident at day 22 in young animals despite the absence of clinical signs but were largely absent from middle-aged animals which still exhibited disease symptoms. At day 40 post inoculation inflammatory infiltrates were sparse in both young and middle-aged animals despite 29% of middle-aged animals being symptomatic. These results suggest that disease chronicity does not correlate with the number of inflammatory lesions. No significant differences were found in the degree of fibrin deposition or demyelination between young and middle-aged or symptomatic and asymptomatic animals. However, astrocytic hypertrophy was found to correlate with disease chronicity, suggesting that astrocytic hypertrophy may play a significant role in the pathophysiology of the disease. Based on these studies, it is proposed that the neurological deficit of chronic EAE in middle-aged animals may be caused by astrocytic hypertrophy with the resolution of this CNS response being a major factor contributing to recovery from the disease.

CHAPTER 6: ANALYSIS OF THE MECHANISMS CONTROLLING AGE-RELATED DIFFERENCES IN EAE SEVERITY AND CHRONICITY

6.1 Introduction

In Chapters 4 and 5 of this study, the pathogenesis of EAE was investigated by specifically looking at age-related differences in the clinical and histological manifestations of disease in Lewis (JC) rats. Although in old rats (over one year old), there was initially a slower and less vigorous onset of disease, aged rats developed a more chronic form of EAE with significantly more deaths than younger rats (Table 4.1). These results suggest that age-related factors can cause a shift in EAE in Lewis (JC) rats from a self-limiting disease to a chronic or relapsing disease of the CNS (refer to Chapter 4). Subsequent studies outlined in Chapter 5 suggested that astrocytic hypertrophy may induce prolonged paralysis in aged animals. Since the observations of disease chronicity in aged animals were obtained from whole animal experiments, EAE chronicity could result from either a modified immune response in aged animals or an inability of the ageing CNS to resolve paralysis resulting from localised inflammatory lesions. In order to distinguish between these two possibilities, this chapter describes experiments where EAE was induced in either young or middle-aged naive recipients by the adoptive transfer of lymphocytes from actively immunised donors. By using such an experimental approach it was hoped to resolve whether disease chronicity in aged animals was a property of the immune system or the CNS milieu.

The production of cytokines is significantly increased during an immune response (Arai et al., 1990) and the infiltration of the CNS by leukocytes in EAE provides a potential source of cytokines. These polypeptides play an important role in the interaction between cells in the immune response and may substantially affect the pathophysiology of the disease. Several investigators claimed to have found a strong correlation between differential cytokine secretion and encephalitogenicity (Powell et al., 1990; Tokuchi et al., 1990), and immunomodulators such as TNF-α, IFN-γ and IL-6 have been implicated in the pathogenesis of demyelinating diseases such as EAE and EAN (Gijbels et al., 1990; Hartung et al., 1990; Ruddle et al., 1990; Selmaj et al., 1991; Chung et al.,1991). Chung et al. (1991) demonstrated that EAE resistant (Brown-Norway) rat astrocytes do not secrete appreciable TNF-α in response to IFN-γ when compared to EAE susceptible (Lewis) rat astrocytes. In Chapter 5, astrocytic hypertrophy was evident in animals exhibiting clinical signs of EAE and it is possible that TNF secreted by cells in the CNS may cause the inflammatory response and initiate disease progression. Enhanced production of TNF may explain the chronic nature of EAE.

The production of cytokines during an immune response has been suggested to induce endocrine-mediated suppression of the inflammatory process (Munck et al., 1984; Besedovsky et al., 1986; Salpolsky et al., 1987; Kroemer et al., 1988) and neuroendocrine-mediated immunoregulation could be relevant in the recovery phase of inflammation in the CNS. With advancing age, the altered functional responsiveness of old cells to glucocorticoid action (Roth, 1979) may be due to a reduction in the numbers of membrane receptors for glucocorticoids (Roth, 1975). Alterations in the neuroendocrine system may also cause functional modifications of immune reactivity (Fabris, 1981) through lymphoid cells which have receptors for hormones (Gavin, 1977; Melmon et al., 1977). For example, steroids have an immunosuppressive effect on EAE (reviewed by Komarek and Dietrich, 1971). Exogenous stress has also been found to suppress EAE in Lewis rats (Levine et al., 1962) and Mason et al. (1990) have proposed that the resistance to EAE seen in some rat strains is due to corticosterone-mediated suppression. In the susceptible Lewis strain, recovery from paralysis correlates with increased levels of corticosterone, and if these rats are adrenalectomised, they are then unable to recover and the disease becomes uniformly fatal, an effect which can be reversed by steroid replacement therapy (Mac Phee et al., 1989). The results of these studies suggest that possibly an exaggerated adrenal response may result in recovery from EAE and differences in the production of corticosterone between young and aged animals may explain differences in disease chronicity.

The anti-inflammatory and immunosuppressive effects of steroids may also be related to their ability to inhibit the production of nitric oxide in inflammatory cells. Conversely, the ability of TNF and related cytokines to cause cell-mediated pathology in CNS diseases may be related to the ability of such cytokines to generate nitric oxide production. Nitric oxide and other nitrogen oxides such as nitrogen dioxide (NO_2), nitrite (NO_2-) and nitrate (NO_3-) are collectively known as reactive nitrogen intermediates (RNIs) (Ding et al., 1988). RNIs may cause tissue damage in host immunopathology (Ding et al., 1988) and are potently anti-microbal and anti-parasitic (Wagner et al., 1988; Green et al., 1990) and have been shown to inhibit protein synthesis (Curran et al., 1991) and mitochondrial respiration (Stehr and Nathan, 1989). RNIs also act as vasodilators (Ignarro, 1987) and have been implicated in microvascular permeability changes (Del Maestro et al., 1981), especially within the CNS (Wei et al., 1985). RNIs, by making the BBB more permeable, may allow other factors into the CNS which contribute to the disease state. RNI production can be augmented by the action of cytokines including TNF-α, IFN-γ, IL-1 and lymphotoxin (Rockett et al., in press) and may be critical in amplifying the cellular response in inflammation (Akira et al., 1990). Nitric oxide is released by both central (Garthwaite et al., 1988) and peripheral (Gillespie et al., 1989; Bulte et al., 1990) neurones and may act as neurotransmitters. It has been suggested that

nitric oxide from extraneural sources could diffuse to nearby neurons and be misinterpreted by the brain as being of synaptic origin and consequently interfere with orderly neuronal transmission (Clark et al., 1992). It is possible that the enhanced production of nitric oxide by CNS cells may initiate inflammation and induce disease chronicity in aged animals.

Another important group of hormones which regulates the immune response are the PGs which suppress immune responses (Goodwin and Webb, 1980). For example, PGE secreted by astrocytes inhibits the ConA response of murine thymocytes (Fontana et al., 1982). Similarly, treatment with precursors of PGs can inhibit the clinical and histological manifestations of EAE in guinea-pigs (Meade et al., 1978) whereas an aggravation of clinical signs of EAE follows indomethacin (an inhibitor of PG synthesis) treatment in rats (Levine and Sowinski, 1977,1980; Ovadia and Paterson, 1982) or guinea-pigs (Bolton and Cuzner, 1980). Since PGE is immunosuppressive, differences in PGE production could be a causative factor in the age-related differences evident in EAE.

It is clear that the age-related mechanisms affecting EAE may be complex since age modifies both the immune and endocrine systems. The heightened disease state found in middle-aged Lewis (JC) rats ensures that it is an appropriate system to test for differences in immunoregulatory factors such as the production of cytokines and the effects of hormone levels on the disease state. The specific aims of this study were:

1) to examine the role of the immune response and the CNS milieu in mediating age-related differences in disease severity and chronicity using the passively transfered disease model of EAE; and
2) in parallel studies to correlate the clinical changes and disease recovery in young and middle-aged animals with the production of the naturally occurring pro-inflammatory mediators TNF and nitric oxide and anti-inflammatory mediators corticosterone and PGE.

6.2 Results

6.2.1 Passive transfer model of EAE

To determine whether the increased chronicity of disease observed in aged animals during active induction of EAE (refer to Chapter 4) was due to a property of the immune response or an inability to resolve CNS paralysis, EAE was passively transferred to naive rats of different ages by intravenous injection of lymphocytes from actively immunised syngeneic donors. Initially, it was found that ConA activated spleen cells from immunised middle-aged rats failed to induce disease in young or middle-aged animals ($50x10^6$ ConA activated spleen cells from immunised middle-aged animals were

tranferred into 5 young and 5 middle-aged animals and none of these animals exhibited clinical signs of EAE). The reasons for this failure were unclear but it may be due to the fact that there was also a 50% reduction in the proliferative response to ConA of spleen cells from aged donors when compared to cells from young donors. Consequently, young animals were used as donors for the passive transfer of disease.

There are substantial differences in the mean weights of young and middle-aged, and male and female rats (Table 6.1), with middle-aged males weighing 35% more than young male rats. A dose response experiment was performed to determine the optimum number of immune spleen cells required to transfer disease to middle-aged male animals. A dose-dependent increase in severity and chronicity was evident in middle-aged rats (Fig. 6.1). When $30x10^6$ cells were transferred, a very mild form of disease was evident. The severity and chronicity of disease increased using higher numbers of cells. As paralysis and a substantial length of disease (7.6 days) was evident in middle-aged animals receiving $50x10^6$ cells, this number of cells was subsequently used to passively transfer disease to young and middle-aged recipients. It was evident that the severity and chronicity of disease using the adoptive transfer model was reduced when compared with active induction of EAE (refer to Chapter 4).

TABLE 6.1: AGE- AND SEX-RELATED DIFFERENCES IN THE MEAN WEIGHTS OF RATS

	N	Male	N	Female
Young (2-3 mths)	3	250 ± 7.7[a]	3	151 ± 0.9
Middle-aged (12-13 mths)	3	387 ± 18.6	3	209 ± 4.6

[a] Weight in grams ± SE

The results from two experiments which examined the age-related differences in severity and chronicity of passively transfered EAE are given in Table 6.2. No significant differences were found for the day of onset of disease in either experiment, with all animals exhibiting clinical signs between 4 to 7 days post inoculation. In experiment one, young animals exhibited a short monophasic episode of disease (lasting 6.7 days) with no

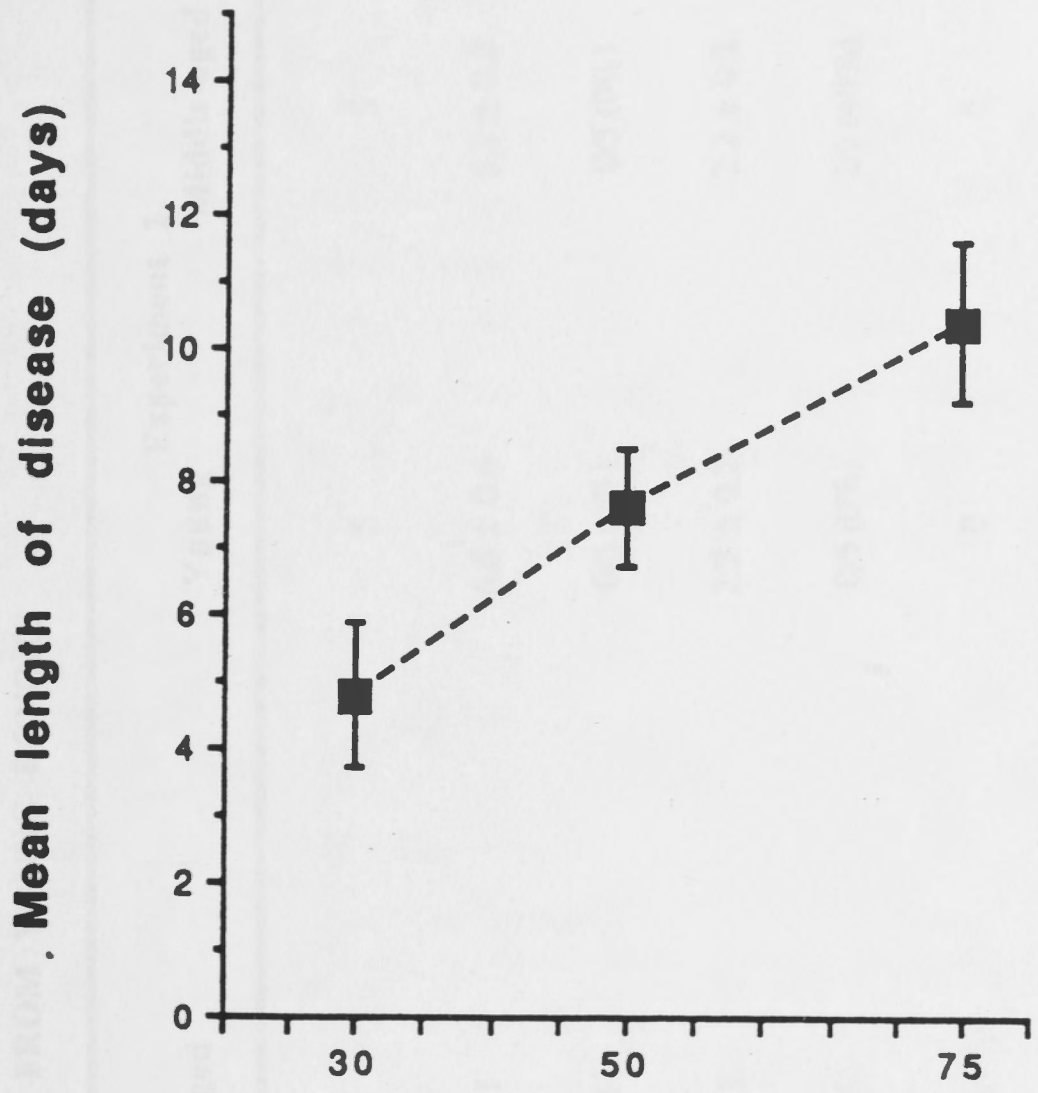


Fig. 6.1: Dose-dependent differences in the chronicity of adoptively transferred EAE in middle-aged male recipients. Animals were assessed for clinical signs of EAE daily and an arbitrary scale of disease severity ranging from 0 to 5 was used as in Fig. 3.2. The length of disease was determined as the number of days clinical signs of EAE were evident. Each data point represents the mean and standard error of 7 animals.

TABLE 6.2

AGE-RELATED DIFFERENCES IN THE CHRONICITY AND SEVERITY OF ADOPTIVELY TRANSFERRED EAE IN MALE RECIPIENTS USING 50X10^6 CON-A EFFECTORS FROM YOUNG DONORS

	Experiment 1		Experiment 2	
	Young	Middle-aged	Young	Middle-aged
Number of Animals	14	15	5	5
Mean day of onset	4.6 ± 0.1[a]	4.6 ± 0.1	6.6 ± 0.6	5.8 ± 0.2
Number of deaths	0/14 (0%)	2/15 (13%)	0/5 (0%)	0/5 (0%)
Mean maximum clinical score	2.7 ± 0.1	4.6 ± 0.1	2.2 ± 0.2	2.2 ± 0.2
Number of relapses[b]	0/14 (0%)	2/13 (15%)	0/5 (0%)	2/5 (40%)
Number of days with hindlimb paralysis	0	0.7 ± 0.2 (9/15)[c]	0	0
Mean length[d] of disease	6.7 ± 0.3	7.6 ± 0.9	3.6 ± 0.7	9.6 ± 0.9

[a] ± se

[b] relapse is defined as a second episode of disease where clinical signs were evident for 2 or more days

[c] number of animals with hindlimb paralysis

[d] number of days clinical signs of EAE were evident

young animals progressing to hindlimb weakness or paralysis, the highest clinical score observed was 3 (ataxia). In contrast, 60% of middle-aged males became paralysed (although paralysis generally only lasted for one day), 13% died and 15% had relapses. In a second experiment, the chronicity of disease was significantly higher in middle-aged rats than in young rats (the mean length of disease was 9.6 days compared with 3.6 days in young animals). Forty percent of middle-aged rats also had relapses in this experiment whereas no young rats exhibited a second episode of disease. These data clearly indicate an age-related increase in the disease severity and/or chronicity of EAE in middle-aged rats when compared with young animals.

As the severity and chronicity of disease was found to be significantly lower in the passive transfer model, active induction was considered the more appropriate model of EAE to assess the relevance of cytokine and hormone levels in the progression of disease.

6.2.2 Measurement of TNF levels

To assess whether the production of TNF correlates with the severity of disease, circulating and local concentrations of TNF were measured by both an ELISA method and a bioassay using MTT to measure cell viability. In this particular model of EAE, no detectable levels of TNF were evident in old and young animals using either of the above methods for the detection of TNF (the limit of detection was 100pg/ml).

TNF and related cytokines are known to cause hypoglycaemia (Clark et al., 1989; Clark et al., 1992), therefore serum glucose levels were determined as an indirect measure of these cytokines. It is evident from Fig. 6.2 that there were no significant differences in glucose levels between young and middle-aged animals or between animals exhibiting clinical signs of EAE and asymptomatic animals at different time intervals during the course of the disease. These results are consistent with no detectable levels of TNF in animals exhibiting clinical signs of EAE and also suggest that other hypoglycaemia-inducing cytokines may not be significantly raised.

6.2.3 Measurement of corticosterone and RNI levels

To assess whether the significant increase in the severity and chronicity of disease in old animals was due to a lack of the production of corticosterone or differences in the production of nitric oxide after active induction, samples were taken at different time intervals from young and middle-aged actively immunised males for the determination of corticosterone and RNI levels. In a preliminary experiment, RNI levels were measured in the blood, CSF and spinal cord of middle-aged male rats at different times after active induction to assess which of these tissues were the best specimens for showing changes in RNI levels. Increased levels of RNI were found in all blood and CSF specimens by day 15 post inoculation, whereas this increase was not observed in

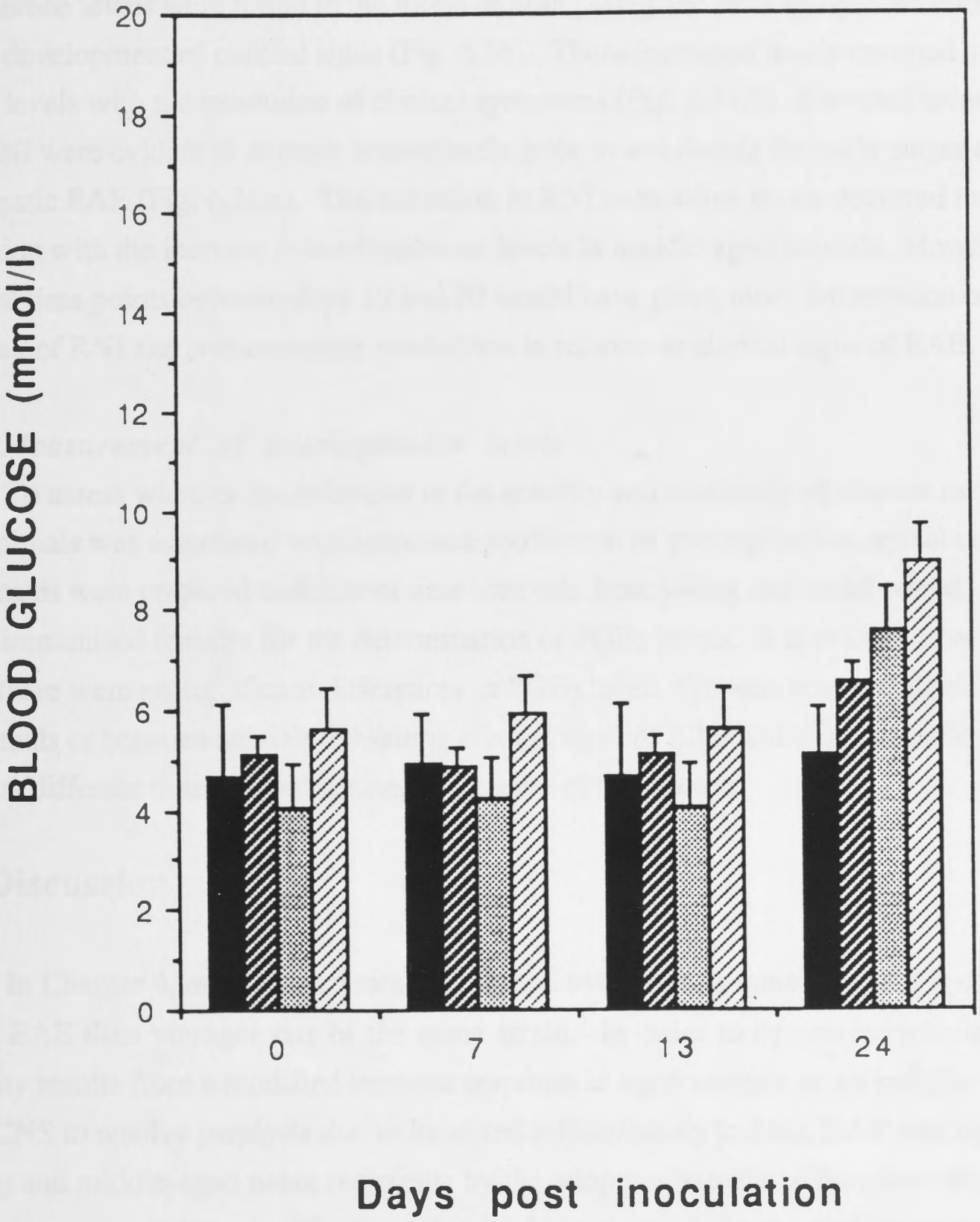


Fig. 6.2: The effect of age on plasma glucose levels during actively induced EAE. Plasma glucose levels (mmol/l) were measured on a Beckman Glucose Analyser in middle-aged (■) and young (▨) male rats inoculated with 50µg MBP in CFA containing 400µg *M. butyricum* or middle-aged (▩) and young (▨) male rats inoculated with normal saline in CFA containing 400µg *M. butyricum*. Each bar represents the mean and standard error of 10 animals in the MBP in CFA groups and 5 animals in the normal saline in CFA groups. At da 24, 3 aged animals had died in the MBP in CFA group.

control animals receiving normal saline in CFA which were measured at day 11 post inoculation (Table 6.3). As blood samples showed significant increases in RNI and these samples can be taken easily without sacrificing the animal, blood was analysed to measure both RNI and corticosterone levels at different times during EAE development. Elevated corticosterone levels were found in the blood of both young and middle-aged animals with the development of clinical signs (Fig. 6.3b). These increased levels returned to baseline levels with the resolution of clinical symptoms (Fig. 6.3a,b). Elevated levels of serum RNI were evident in animals immediately prior to and during the early stages of symptomatic EAE (Fig. 6.3a,c). The reduction in RNI to baseline levels occurred in conjunction with the increase in corticosterone levels in middle-aged animals. However, additional time points between days 10 and 20 would have given more information on the exact peak of RNI and corticosterone production in relation to clinical signs of EAE.

6.2.4 Measurement of prostaglandin levels

To assess whether the reduction in the severity and chronicity of disease in young animals was associated with increased production of prostaglandins, spinal cord homogenates were prepared at different time intervals from young and middle-aged actively immunised females for the determination of PGE_2 levels. It is evident from Fig. 6.4 that there were no significant differences in PGE_2 levels between young and middle-aged animals or between animals exhibiting clinical signs of EAE and asymptomatic animals at different time intervals during the course of the disease.

6.3 Discussion

In Chapter 4, middle-aged rats were found to develop a more severe and chronic form of EAE than younger rats of the same strain. In order to determine whether the chronicity results from a modified immune response in aged animals or an inability of an ageing CNS to resolve paralysis due to localised inflammatory lesions, EAE was induced in young and middle-aged naive recipients by the adoptive transfer of lymphocytes from actively immunised donors. The data clearly demonstrated increased disease severity and/or chronicity of EAE in middle-aged rats when compared with young animals. These results suggested that disease chronicity in middle-aged animals was a property of the CNS milieu rather than a property of an ageing immune system.

In parallel studies the naturally occurring pro-inflammatory mediators TNF and nitric oxide and anti-inflammatory mediators corticosterone and PGE were examined in young and middle-aged animals. TNF-α was not detected in young or middle-aged rats throughout the course of the disease and no significant differences were found for PGE_2 levels between young and middle-aged animals or between symptomatic or asymptomatic

TABLE 6.3

CIRCULATING AND LOCAL REACTIVE NITROGEN INTERMEDIATE LEVELS IN MIDDLE-AGED MALE RATS DURING THE COURSE OF EAE

DPI[a]	N	Blood (μM)	N	CSF (μM)	N	Spinal Cord (μM)
0	6	2.2 ±0.5[b]	3	41.6 ±8.9	6	2.2 ±0.5
11	3	1.6 ±0.4	3	103.9 ±26.3	3	4.8 ±2.3
15	6	19.6 ±5.8	2	272.0 ±49.4	6	3.1 ±0.9
Control NS+CFA Day 11	6	1.9 ±0.4	3	63.5 ±3.3	6	2.2 ±0.3

a Days post inoculation
b ± SE

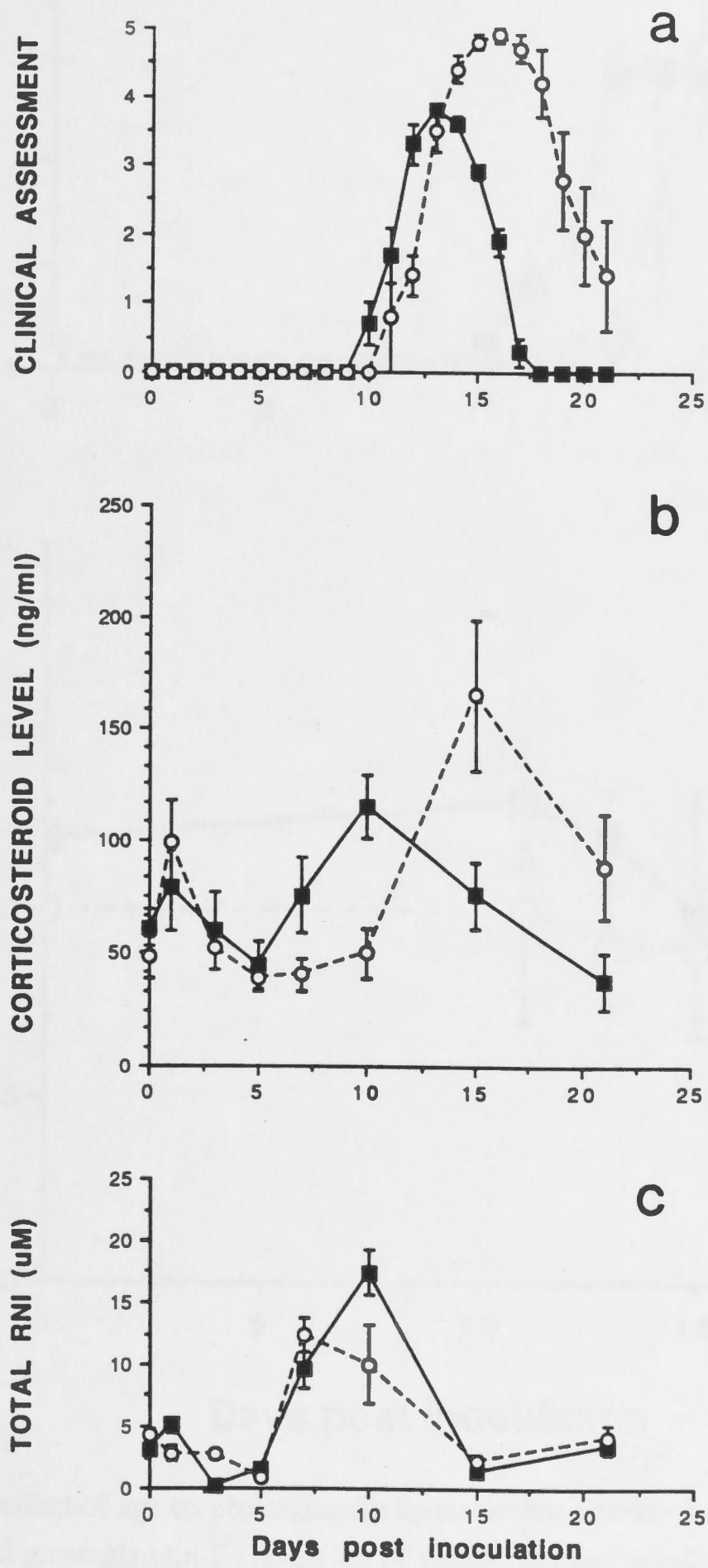


Fig. 6.3: The effect of age on serum corticosterone and reactive nitrogen intermediate levels during actively induced EAE. Clinical profiles (a), and changes in serum corticosterone (b) and reactive nitrogen intermediate (c) levels of young (■) and middle aged (○) male rats inoculated with 50μg MBP in CFA containing 400μg *M. butyricum*. Animals were assessed for clinical signs of EAE daily and an arbitrary scale of disease severity ranging from 0 to 5 was used as in Fig. 3.2. Corticosterone levels were measured by RIA and reactive nitrogen intermediate levels were determined by an assay based on the reduction of nitrate by a CdCu complex coupled to Griess reagent for azochromaphoretic detection. The results are expressed as micromolar concentrations of total RNI (the sum of nitrite and nitrate) concentrations. Each data point represents the mean and standard error of 12 animals. At day 21, 5 aged animals had died.

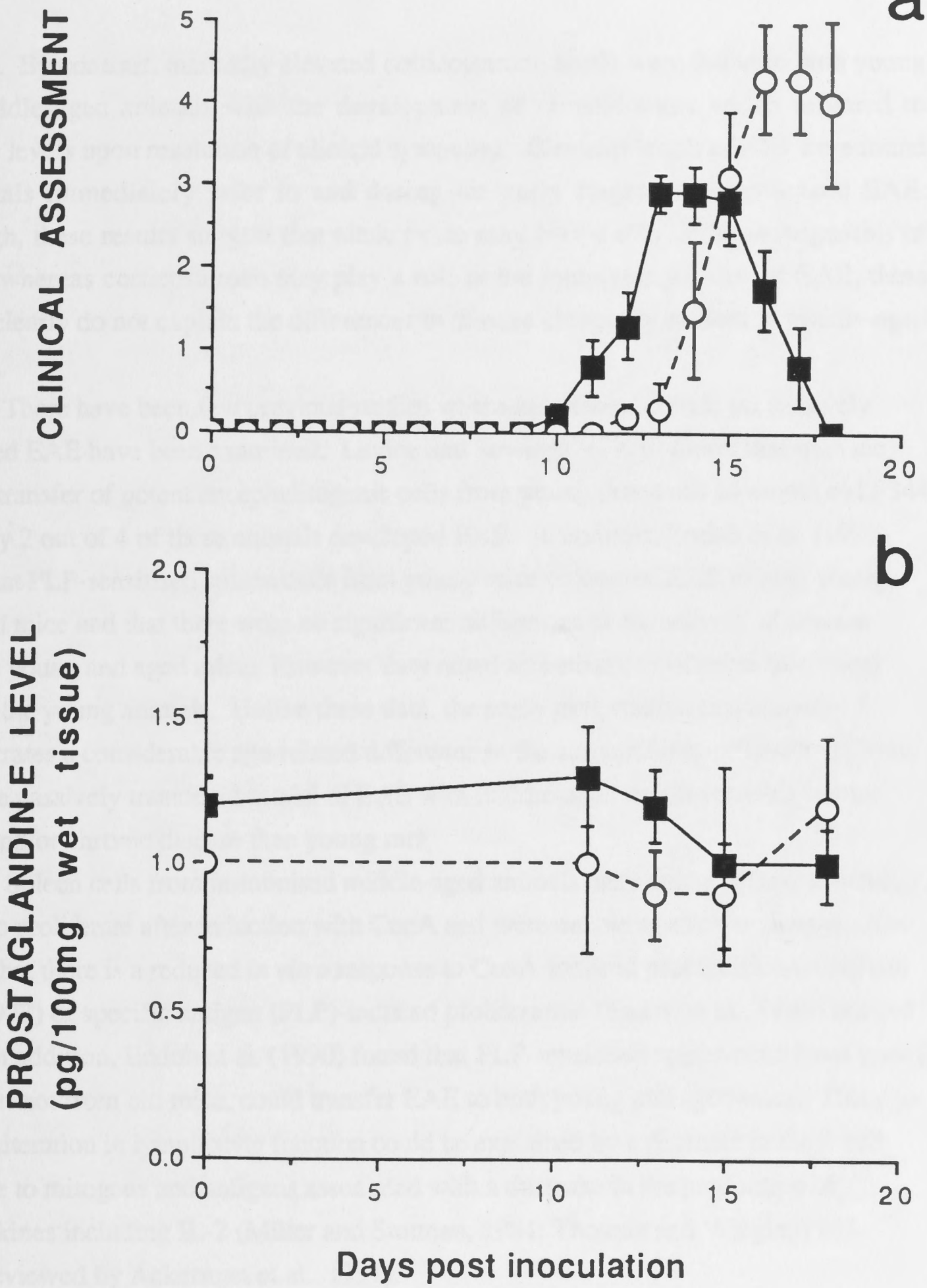


Fig. 6.4: The effect of age on prostaglandin levels during actively induced EAE. Clinical profiles (a) and prostaglandin-E_2 levels (b) of young (■) and middle aged (O) male rats inoculated with 50µg MBP in CFA containing 400µg *M. butyricum*. Animals were assessed for clinical signs of EAE daily and an arbitrary scale of disease severity ranging from 0 to 5 was used as in Fig. 3.2. Prostaglandin levels were measured by RIA using a PGE_2 (^{125}I) assay system. Each data point represents the mean and standard error of 3 animals.

animals. By contrast, markedly elevated corticosterone levels were found in both young and middle-aged animals with the development of clinical signs which returned to baseline levels upon resolution of clinical symptoms. Elevated levels of RNI were found in animals immediately prior to and during the early stages of symptomatic EAE. Although, these results suggest that nitric oxide may play a role in the pathogenesis of disease whereas corticosterone may play a role in the immunoregulation of EAE, these factors clearly do not explain the differences in disease chronicity evident in middle-aged animals.

There have been few previous studies where age-related effects on passively transfered EAE have been examined. Levine and Sowinski (1976) found that with the passive transfer of potent encephalitogenic cells from young donors to 24 month old F344 rats, only 2 out of 4 of these animals developed EAE. In contrast, Endoh et al. (1990) found that PLP-sensitised spleen cells from young mice transfered EAE to both young and aged mice and that there were no significant differences in the severity of disease between young and aged mice. However they noted an earlier day of onset of clinical signs in the young animals. Unlike these data, the study presented in this chapter demonstrates a considerable age-related difference in the susceptibility of Lewis (JC) rats using the passively transfered model of EAE with middle-aged rats developing a more severe and/or chronic disease than young rats.

Spleen cells from immunised middle-aged animals were found to have a reduced ability to proliferate after induction with ConA and were unable to transfer disease. It is known that there is a reduced *in vitro* response to ConA-induced proliferation (Abraham et al., 1977) or specific antigen (PLP)-induced proliferation (Endoh et al., 1990) in aged mice. In addition, Endoh et al. (1990) found that PLP-sensitised spleen cells from young mice, but not from old mice, could transfer EAE to both young and aged mice. This age-related alteration in lymphocyte function could be explained by a decrease in the T cell response to mitogens and antigens associated with a decrease in the production of lymphokines including IL-2 (Miller and Stutman, 1981; Thoman and Wiegle, 1981, 1982; reviewed by Ackerman et al., 1991).

Despite no TNF-α being detected in animals with EAE in this study, TNF-α has been implicated in the pathogenesis of EAE (Ruddle et al., 1990; Selmaj et al., 1991; Chung et al.,1991). TNF has been found to have a paradoxical effect on autoaggressive processes depending on the administration schedule and the experimental system (Teuscher et al., 1990; Ruddle et al., 1990; Selmaj et al., 1991). TNF production may possibly be involved in other models of disease where an enhanced immune response is evident, eg. in a model where pertussis is added to the inoculum.

Since PGE is immunosuppressive, it was hypothesised that its production could be a causative factor in some of the age-and sex-related differences evident in EAE. The

regulatory role of PGs in EAE was suggested by studies in which guinea-pigs fed on large amounts of dietary polyunsaturated fatty acids (which are precursors of PGs) did not develop clinical or histological manifestations of EAE (Meade et al., 1978). This inhibitory effect of polyunsaturated fatty acids can be reversed by *in vivo* administration of indomethacin (an inhibitor of PG synthesis) (Hughes et al., 1980). However, in the study reported in this chapter, there were no significant differences in PGE_2 levels between young and middle-aged animals or between symptomatic and asymptomatic animals at different time intervals during the course of the disease. In contrast, significant changes in the PGE content of CNS tissue during the induction and progression of EAE has been found in guinea-pigs (Bolton et al., 1984a) and Lewis rats (Bolton et al., 1984b). PGE was found in these studies to increase before the appearance of clinical signs and to rise until approximately 12 days post inoculation when they returned to normal levels. One explanation for the drop in PGE observed in these studies could be that glucocorticoid hormones secreted from the adrenal cortex or administered as a drug curtail the availability of arachidonic acid (Claman, 1983) and possibly exogenously block PG production. However, in this model of EAE, no increase in PGE_2 levels were found. The explanation for these differences is unclear.

Elevated corticosterone levels were found in both young and middle-aged animals with the development of clinical signs. These increased levels returned to baseline levels with the resolution of clinical symptoms (Fig. 6.3a,b). It has been suggested that recovery from EAE results from an exaggerated adrenal response (Levine et al., 1962; MacPhee et al., 1989; Mason et al., 1990). For example, stress which has been shown to cause an increase in the production of adrenocortical hormones, was found to suppress EAE in Lewis rats (Levine et al., 1962). Subsequently, it was found that if Lewis rats were adrenalectomised, they were unable to recover and the disease became lethal, an effect which was reversed by steroid replacement therapy (Mac Phee et al., 1989). However, adrenalectomy performed 12 to 14 days after induction of EAE did not result in the development of disease suggesting that the long-term refractory period found in recovered rats does not depend on corticosterone-mediated suppression (MacPhee et al., 1989). Mason et al. (1990) have also proposed that the resistance to EAE seen in some strains is due to corticosterone-mediated suppression. The PVG strain of rat, which has significantly higher basal levels of corticosterone than those in Lewis rats (Mason et al., 1990) is not susceptible to EAE. However, this strain develops severe disease after adrenalectomy with steroid replacement therapy preventing the fatal progression of disease. Griffin and Whitacre (1991) found that Lewis rats had low basal levels of circulating corticosterone, and high numbers of CD4+ T cells compared with F344 rats and they suggested that these factors may play a causative role in the susceptibility of Lewis rats to autoimmune diseases such as EAE. In the study reported in this chapter, it

was hypothesised that an age-related decline in the production of corticosterone may cause the chronicity of disease evident in middle-aged animals. However, it was found that middle-aged animals exhibited a substantial increase in corticosterone levels during the course of disease. Although a reduction in the concentration of membrane receptors for glucocorticoids has been found with advancing age (Roth, 1975), circulating corticosterone levels as measured by RIA have previously been found to progressively increase with age (Iams and Wexler, 1979). In the study presented in this chapter, an increase in corticosterone production was associated with an increase in disease severity and the disease recovery followed the peak in corticosterone levels. Whether the elevated levels of corticosterone play an immunoregulatory role or are due to the stress caused by the clinical symptoms of EAE is unclear and needs further study.

Elevated levels of RNI were found in young and middle-aged animals immediately prior to and during the early stages of symptomatic EAE. RNIs have vasodilatory effects in the brain and have been implicated in microvascular permeability changes (Del Maestro et al., 1981) and may be critical in amplifying the cellular response in localised forms of inflammation (Akira et al., 1990). A critical event in the development of encephalomyelitis is the breakdown of the BBB and guinea pigs and monkeys immunised with CNS endothelial membrane fractions uncontaminated with myelin proteins can cause an EAE-like disease consisting of paralysis and demyelination (Tsukada et al., 1987). This result suggests that an immune disturbance of the BBB is sufficient to cause EAE and nitric oxide may be involved in permeability of the BBB. In the study presented in this chapter, the reduction in RNI to baseline levels occurred in conjunction with the increase in corticosterone levels in middle-aged animals though this was not evident in young animals. Glucocorticoids have previously been found to inhibit the production of nitric oxide in macrophages (Di Rosa et al., 1990) and endothelial cells (Radomski et al., 1990; Knowles et al., 1990). Macrophages, which express high affinity receptors for glucocorticoids (Werb et al., 1978) play a significant role in the inflammatory process in EAE (Panitch and Ciccone, 1981; Brosnan et al., 1981; Killen and Swanborg, 1982a). The anti-inflammatory and immunosuppressive effects of steroids may be related to the ability to inhibit the production of nitric oxide in inflammatory cells (Knowles et al., 1990; di Rosa et al., 1990; Moncada and Palmer, 1991). Additional time points between days 10 and 20 would have given more information on the exact peak of RNI and corticosterone production in relation to clinical signs of EAE.

The results presented in this chapter, are consistent with the hypothesis that nitric oxide may play a role in the pathogenesis of disease whereas corticosterone may be involved in the immunoregulation of the disease. However, these factors do not explain the differences in chronicity of disease evident in middle-aged animals. Possibly, as

suggested in Chapter 5, astrocytic hypertrophy may induce prolonged paralysis in aged animals.

6.4 Summary

In Chapter 4, middle-aged rats were found to develop a more severe and chronic form of EAE than younger rats of the same strain. In order to determine whether the chronicity results from a modified immune response in middle-aged animals or an inability of an ageing CNS to resolve paralysis resulting from localised inflammatory lesions, EAE was induced in young and middle-aged naive recipients by the adoptive transfer of lymphocytes from actively immunised donors. It was found that middle-aged recipients developed more severe disease than young recipients. Based on these observations it was concluded that disease chronicity in middle-aged animals is a property of the CNS milieu rather than a property of an ageing immune system. In parallel studies the naturally occurring pro-inflammatory mediators TNF and nitric oxide and anti-inflammatory mediators corticosterone and PGE were examined in young and middle-aged animals using the actively induced model of EAE. No detectable levels of TNF-α were evident in middle-aged and young rats during the course of disease and no significant differences in PGE_2 levels between young and middle-aged animals or between symptomatic and asymptomatic animals were found. By contrast, markedly elevated corticosterone levels were found in both young and middle-aged animals with the development of clinical signs which returned to baseline levels with the resolution of clinical symptoms. Elevated levels of RNI were evident in animals immediately prior to and during the early stages of symptomatic EAE. Although, these results suggest that nitric oxide may play a role in the pathogenesis of disease, whereas corticosterone may play a role in the immunoregulation of the disease, these factors cannot explain differences in disease chronicity evident in middle-aged animals.

CHAPTER 7: DISCUSSION AND CONCLUDING REMARKS

EAE, a major applied model for MS research, is also an excellent model for studying interactions between the immune system and the CNS. The specific aims of this study were to ascertain how T cells, which are normally found in low numbers in the CNS, localise during the inflammatory response and as MS and EAE are both age-dependent diseases, to establish the clinical, histopathological and immunoregulatory changes which occur in aged animals suffering from EAE.

7.1 T cell entry into the CNS

Lymphocytes are normally found in low numbers in the CNS. Migration of T cells to the CNS and their cellular distribution in EAE lesions is well documented. How their initial entry is achieved during EAE in the absence of gross damage to the vascular endothelium is unknown. This study addressed the hypothesis put forward by Wekerle et al. (1986) that "activated" lymphocytes of any specificity enter the CNS but only those with neuro-antigen specificity persist and cause pathology. Using EAE in the rat as a model of CNS inflammation, H33342-labelled activated and quiescent T cells with different antigen specificities were tested for their ability to accumulate in different regions of the spinal cord and in other organs at varying times post inoculation. The data presented in this study strongly supported the Wekerle hypothesis as T cell lines specific for MBP, PPD, or OA all entered the spinal cord, if first activated with their specific antigen, but only MBP-specific cell lines accumulated. OA- and PPD-specific T cell lines were used because the antigen-specificities of these lines are non-specific to the CNS and cause no known illness or inflammation in syngeneic rats.

The state of activation of the MBP-specific cells was also found to be critical for the induction of disease. Non-activated MBP-specific T cells failed to accumulate in the spinal cord to a substantial degree and did not cause clinical signs of EAE. These results are consistent with the earlier finding that quiescent cells do not cause disease (Panitch and Ciccone, 1981; Ben-Nun et al., 1981; Holda et al., 1980; Peters and Hinrichs, 1982; Naparstek et al., 1983) and lack the elevated levels of heparanases (Naparstek et al., 1984) which facilitate penetration of the cells through the CNS endothelium and into the inflammatory site (Willenborg and Parish, 1988; Willenborg et al., 1989a, 1989b).

MBP-specific T cell accumulation was found to be similar throughout the different regions of the spinal cord during the first 3 days post inoculation Histologically, lesions were also apparent throughout the spinal cord at days 4 and 7 post inoculation and there was marked perivascular inflammation evident in all regions of the cord. As EAE is

a disease consisting of an ascending paralysis with clinical signs attributed to disturbances of the lumbar-sacral areas of the cord, it is possible that either lower spinal cord nerve damage is more likely to result in the clinical signs of disease or otherwise due to the caudally increasing length of the spinal roots (Waibl, 1973), the probability of lesion formation progressively increases in a caudal direction.

The substantial increase in accumulation of the antigen-activated MBP-specific T cells between days one and four was much more than could be accounted for by either random entrance of activated cells or by the replication of the cells *in situ* once they had entered the parenchyma. One possible explanation is that the MBP-specific cell line is being recruited in a non-specific fashion following the initial entrance of specific cells and their subsequent interaction with antigen. If this is the case then one would expect that antigen-activated, non-neuroantigen specific T cells could be recruited into the CNS if injected along with activated MBP-specific cells. This is indeed what happened. The CNS accumulation of an OA-activated, OA-specific T cell line increased 15-20 fold when injected simultaneously with a MBP-activated, MBP-specific T cell line. However, there was a delay in CNS accumulation of the OA-specific T cells, suggesting the need for the MBP-specific T cells to first establish an inflammatory response.

To examine the host contribution to passively induced EAE, the entrance and accumulation of activated MBP-specific T cells was assessed in irradiated recipients. Radiation has been reported to have varying effects on the induction of EAE (Levine et al., 1969) with variations probably being related to the species studied, the dose, the timing and the type of radiation. Sedgewick et al. (1987) reported that regardless of the status of the recipient (irradiated or not), clinical signs were induced when animals received MBP-reactive T cells although the number of infiltrating leukocytes was reduced in irradiated animals. In this study, activated MBP-specific T cells accumulated in the spinal cord of irradiated rats but to a lesser extent than in non-irradiated recipients. There was also a delay in the kinetics of accumulation by about two days which agrees with the altered kinetics of clinical signs. Two possible explanations for these results would be that irradiation may directly affect the endothelium in a way that makes it less adhesive or alternatively the recruitment of "non-specific" host cells may in turn act as recruiting cells in further promotion of the inflammatory response. In this study, the failure of non-specific T cells to accumulate in the CNS rules out the possibility of extensive radiation-induced changes in the vasculature allowing non-specific cellular infiltration.

Differences in the size and distribution of inflammatory lesions between irradiated and non-irradiated recipients were observed histologically in spinal cord sections. In this study, as in that of Sedgewick et al. (1987), the number of inflammatory

cells in the spinal cord of irradiated animals was reduced compared to non-irradiated recipient animals. The correlation between the number of lesions in the CNS and flaccid paralysis has been found to be poor (Hoffman et al., 1973; Raine, 1980; Simmons et al., 1982, 1984), and possibly other mechanisms of dysfunction cause clinical symptoms of EAE. Considerable hemorrhage and the exposure of collagen, a component of the extracellular matrix, was found in irradiated recipients in this study, suggesting that the integrity of the vasculature had been affected. Sedgewick et al. (1987) also found that disease in irradiated recipients was associated with substantial hemorrhagic lesions in the spinal cord and brain stem. In addition, a complete absence of hemorrhagic lesions was reported in irradiated recipients receiving OA-reactive T cells, suggesting that the vascular damage was caused by the MBP-reactive T cells. In support of this hypothesis, the clinical signs of EAE and hemorrhage were preventable by the administration of anti-CD4 mAbs, leading to the conclusion that the ability to repair damage has been compromised in irradiated animals.

An important observation in the study described in this thesis, was that astrocytic proliferation was evident in irradiated and non-irradiated animals exhibiting advanced clinical signs of EAE. The astrocytic response to injury of the CNS is a process called reactive gliosis (Miller et al., 1986). Scars are formed by astrocytes extending numerous processes that become larger and have a substantial increase in glial filaments (Maxwell and Kruger, 1965; Eng, 1985). The proliferating astrocytes engage in phagocytosis (Noske et al., 1982; Kandel, 1985; Eng, 1985), and consequently astrocytes remove debris and help seal-off damaged brain tissue after neuronal death or injury (Kandel, 1985). This encroachment of astrocytes displaces presynaptic terminals and results in the damaged neurones receiving reduced synaptic input, with the evoked excitatory presynaptic potentials being smaller in amplitude (Kandel, 1985). These results suggest that astrocytes could be important in the development of clinical signs of EAE with the loss of nerve transmission which results in paralysis possibly being due to astrocytic proliferation slowing the conduction of fibers.

7.2 Age related changes in EAE

It is clearly established that EAE is mediated by class II MHC antigen-restricted T cells (Pettinelli and McFarlin, 1981; Zamvil et al., 1985). However, the actual mechanisms which initiate the CNS entry of T cells and which result in the subsequent CNS damage produced by the ensueing inflammatory response remain unclear. In this context, it was of considerable interest to determine whether advanced age, which is associated with a decline in T cell-mediated immunity, protects against EAE, a T cell mediated disease.

Although EAE is usually a monophasic self-limiting disease in rats, recurrent EAE has been previously reported in aged (6 months old, Mc Farlin et al., 1974 and 13 months old, Ben-Nun et al., 1980) female Lewis rats. Kallen and Logdberg (1982) also reported there was an age-effect on the susceptibility of Lew/Mol (a nearly EAE resistant Lewis substrain) rats. In contrast, an age related decline in susceptibility to EAE has been demonstrated by some researchers in mice (Endoh et al., 1990) and F344 rats (Levine and Sowinski, 1976). Due to the array of pathologic lesions found in aged F344 rats (Sass et al., 1975; Levine and Sowinski, 1976), it appears that F344 rats are not a good model for assessing age-related factors. In this study, the pathogenesis and immunoregulation of EAE was investigated by specifically looking at age-related differences in Lewis (JC) rats, a rat strain which does not suffer from a multiplicity of age-related illnesses.

The results showed that aged Lewis (JC) rats develop a more chronic form of EAE with significantly more deaths than younger rats of the same strain. Although there was a slower and less vigorous response initially in aged animals, consistent with an age-dependent decline in T cell-mediated immunity, paradoxically the disease chronicity in middle-aged and geriatric rats was significantly higher. Young animals exhibited a short monophasic episode of disease, had a low death rate and only a few animals exhibited a mild second episode of disease. These results suggest there is a deficit in the resolution of paralysis resulting from CNS inflammatory lesions in older animals. The study also revealed that male rats exhibited increased severity and chronicity of disease compared with females of the same age. However, sterilisation of females did not have a significant effect on disease profiles.

On the other hand, studies reported in this thesis demonstrated no significant differences between young and aged animals in the production of MBP-specific antibody. Furthermore, MBP-specific antibody production did not correlate with onset, severity or relapse of EAE in both young and middle-aged animals. These are similar results to those found in previous studies where MBP-specific antibody levels were determined in young animals (Lisak et al., 1969; Lennon et al., 1971; Tabira and Endoh, 1985). Finally, when EAE was induced in young and middle-aged naive recipients by the adoptive transfer of lymphocytes from actively immunised donors, the data clearly demonstrated increased disease severity and/or chronicity of EAE in middle-aged rats when compared with young animals. These results suggested that disease chronicity in middle-aged animals was a property of the CNS milieu rather than a property of an ageing immune system.

7.3 Astrocytes play a central role in EAE chronicity

In order to determine whether the age-related differences in the clinical manifestation of EAE could be attributed to gross differences in the histopathology of the disease in middle-aged animals, the inflammatory lesions were quantified, and the degree of fibrin deposition, demyelination and astrocytic hypertrophy was compared.

It has previously been found in EAE, that the correlation between the number of lesions in the CNS and flaccid paralysis is poor (Hoffman et al., 1973; Raine, 1980; Simmons et al., 1982, 1984). Similarly, in this study, it was evident that the persistence of inflammatory lesions found in young animals at day 22 was not associated with chronicity of disease. In addition, the resolution of lesions in middle-aged animals at days 22 and 40 post inoculation suggests that the persistence of the neurological deficit found in middle-aged animals at these time points is not due to inflammatory lesions.

Although fibrin has previously been found to be deposited in lesions of EAE (Oldstone and Dixon, 1968; Paterson, 1976; Rauch et al., 1978) and has been suggested as a cause of paralysis in EAE (Paterson, 1976), no significant fibrin deposition above background was found in this model of EAE in young and middle-aged or symptomatic and asymptomatic animals using both light and electron microscopic methods for the detection of fibrin. The reason for this discrepancy with earlier studies is unclear. However the results clearly indicate that fibrin deposition does not cause the chronicity of the neurological deficit found in middle-aged animals using this particular model of EAE.

In this model of EAE, demyelination was not apparent in young and middle-aged, or symptomatic and asymptomatic animals. This finding is consistent with earlier reports where demyelination was found to be sparse or absent in acute models of EAE, particularly if MBP is the inoculum (Hoffman et al., 1973; Lassman and Wisniewski, 1979; Panitch and Ciccone, 1981; Raine et al., 1981; Simmons et al., 1981, 1983; White, 1984; Kerlero de Rosbo et al., 1985). It has also been suggested that recovery from EAE occurs too rapidly to be attributed to remyelination (McDonald, 1974a,b; Panitch and Ciccone, 1981; Simmons et al., 1981). Demyelination has usually been found in animals with a more chronic disease process (Tabira and Sakai, 1987) and it was hypothesised that the more chronic form of disease in middle-aged animals may be due to demyelination. However, this was clearly not the case. The results presented in this thesis suggest that generalised demyelination is not the cause of the persistent neurological deficit found in middle-aged animals in this model of EAE, although electron microscopic studies would be necessary to discount highly localised demyelination in the spinal cord ventral roots as suggested by Pender (1988b).

In this study astrocytic hypertrophy was found to increase during the development of disease and persist in symptomatic middle-aged animals. Astrocytic proliferation was also found in irradiated and non-irradiated animals exhibiting advanced clinical signs of EAE. These results suggest that astrocytes may play an important role in the pathogenesis of EAE. Fibrillary astrogliosis has previously been found to be one of the preclinical changes evident in EAE (Field, 1961; Bubis and Luse, 1964; Lampert, 1967). Gliosis is also a prominant feature in the chronic model of EAE in the guinea pig and mouse (Linington et al., 1984; Raine, 1983; Smith et al., 1984, 1985). In the studies presented in this thesis, the intensity of staining increased with time post inoculation with reactive astrocytes being distributed throughout the tissue and decreasing in their intensity of staining as clinically evident disease subsided. Similarly, other investigators have also shown that in acute EAE in the Lewis rat enhanced immunocytochemical staining of GFAP is evident 10 to 12 days post inoculation with staining for GFAP increasing with time (Smith et al., 1983; Goldmuntz et al., 1986; Cammer et al., 1990).

An intriguing question arising from these observations is how astrocytic hypertrophy can inhibit nerve conductance. In this context, it is possible that the astrocytic hypertrophy evident in middle-aged animals results in chronicity of disease by the long astrocytic processes interfering with synaptic input. Astrocytes that surround the synaptic region, also have a high affinity for certain neurotransmitters such as gamma aminobutyric acid and serotonin (Bowman and Kimelberg, 1984; Kandel and Schwartz, 1985; Kimelberg and Katz, 1985). Thus, it is possible that astrocytes, by their ability to bind and internalise neurotransmitters, may reduce local concentrations of specific neurotransmitters and consequently interfere with the conduction of impulses. Finally, it could be argued that astrocytes interfere with neurotransmission via unknown mechanisms, such as secretion of inhibitors of neurotransmission.

7.4 Effect of age on production of inflammatory mediators

Although EAE chronicity in aged animals is most likely due to astrocytic hypertrophy, it was unclear which factors both initiate and suppress the astrocytic response. It is known that CD4+ T cells are cytotoxic for MBP-presenting astrocytes *in vitro* (Sun and Wekerle, 1986) and possibly CD4+ T cells in inflammatory lesions cause damage to astrocytes within the CNS, resulting in the formation of astrocytic hypertrophy and consequently in nerve conduction defects and clinical symptoms of EAE. Another possibility is that the naturally occurring pro-inflammatory mediators TNF and nitric oxide and anti-inflammatory mediators corticosterone and PGE regulate the astrocytic response. No TNF-α was detected in animals with EAE in this study. However, TNF production may

possibly be involved in other models of disease where an enhanced immune response is evident, eg. in a model where pertussis is added to the inoculum. In addition, there were no significant differences in PGE_2 levels between young and middle-aged animals or between symptomatic and asymptomatic animals at different time intervals during the course of the disease.

Based on earlier studies by others (Levine et al., 1962; MacPhee et al., 1989; Mason et al., 1990), it could be hypothesised that an age-related decline in the production of corticosterone may cause the chronicity of disease evident in middle-aged animals. However, it was found that middle-aged animals exhibited a substantial increase in corticosterone levels during the course of disease. This increase in corticosterone production was associated with an increase in disease severity and the disease recovery followed the peak in corticosterone levels. Whether the elevated levels of corticosterone play an immunoregulatory role or are due to the stress caused by the clinical symptoms of EAE is unclear and needs further study. Nevertheless, data from this study clearly eliminated corticosterone as a regulator of disease chronicity. When RNI levels were assessed, the reduction in RNI to baseline levels occurred in conjunction with the increase in corticosterone levels in middle-aged animals though this was not evident in young animals. Additional data points are required to establish whether this difference is of relevance to age-related differences in EAE induction. However, as with corticosterone, it seems unlikely that RNI plays any role in disease chronicity.

Collectively, the data suggests that none of the naturally occurring inflammatory mediators examined are associated with prolonged astrocytic hypertrophy. Nevertheless, the possibility still remains that the pro-inflammatory mediators TNF and RNI may initiate the astrocytic response.

7.5 Relevance to MS

In this study, a memory response was evident in aged animals 13 months after receiving MBP-specific effector T cells, suggesting that an early etiological event may set up an autoimmune carrier state which can persist even into middle-age. These results support the hypothesis put forward by many researchers that MS is caused by a viral infection occurring in childhood or adolescence which induces prolonged immunological memory to CNS antigens (Wege et al., 1984; Tardieu et al., 1984; Waksman, 1988). Coupled with the observation in this study that EAE is more severe in aged animals, in MS an early etiological event may set up an autoimmune carrier state which can persist even into middle-age and be reactivated with chronic disease resulting due to the aged persons inability to resolve inflammation effectively.

MS is a disease in which intense fibrous astrogliosis is a prominant feature (Raine, 1983, 1984) and it is possible that astrocytes contribute to tissue damage by acting as either auxillary or effector cells. Active and replicating astrocytes are prominant in the MS lesion (Adams, 1983) where they fill areas formerly occupied by myelinated axons (Raine, 1985). The demonstration in this thesis that astrocytic hypertrophy is associated with neurological dysfunction, in the absence of demyelination, raises the interesting possibility that astrocytic hypertrophy may play a significant role in both acute and chronic MS, independent of demyelination.

7.6 Future Work

An important unanswered question is the nature of the factor(s) which control initial entry of antigen-specific lymphocytes into the CNS. When comparing the studies of Naparstek et al. (1983), Hickey et al. (1991), Lightman et al. (1987) and my own (all of which used T cell lines in a rat model) it is evident that where accumulation of antigen specific T cells is reported, the cell lines were activated with specific antigen, but when ConA was used to activate the cell lines antigen specific T cells did not accumulate. In addition, both activated non-neurospecific T cell lines (OA- and PPD-specific) enter the lower spinal cord and persist at a low level. There was a difference in the absolute numbers of cells observed for the two cell lines, with the PPD-specific T cells being more numerous. One possible explanation for this difference is that the two cell lines differed in their degree of "activation" and hence their ability to enter the CNS parenchyma. This brings up the question of what exactly "activation" represents. This is currently unknown but it has been suggested that activation translates to the expression of enzymes on the lymphocyte surface that facilitate the degradation of the sub-endothelial basement membrane and extracellular matrix thus promoting emigration (Willenborg and Parish, 1988; Willenborg et al., 1989a, 1989b). There are, of course, a number of other interpretations of what "activation" represents such as the up-regulation of various lymphocyte cell adhesion molecules with resultant increased adhesion to endothelial cells (Cannella et al., 1990; Sobel et al., 1990), or the ability of activated but not non-activated cells to produce a given range of cytokines (Powell et al., 1990). Comparing specific antigen-activated with ConA-activated T cells with respect to their ability to express enzymes, adhesion molecules or various cytokines may provide valuable information.

Studies presented in this thesis suggest that in contrast to a role in healing CNS injury, astrogliosis may produce pathological effects by interfering with neuronal conduction and regeneration. Therefore, further studies are needed to clarify the causes of the astrocytic hypertrophy which possibly results in the neurological deficit found in this model of EAE. Of considerable interest is the nature of the factor(s) which initiate the astrocytic response. Are certain inflammatory cytokines involved, are critical

interactions between lymphocytes and CNS elements required and are particular T cell subsets involved? Similarly, electron microscopic studies may possibly reveal the encroachment of astrocytic processes on neurones and how this encroachment correlates with symptoms of disease. *In vitro* models also could be used to examine the interaction between astrocytes and neurones and to determine whether inhibition of nerve conductance by astrocytes is due to simple physical encroachment or to the inhibition of neurotransmitter action. Investigation of factors which downregulate astrocytic hypertrophy and whether such factors are lacking in the ageing CNS is another important area of study.

In conclusion, this study clearly shows that astrocytes are potential targets for therapeutic intervention as they affect neurophysiological function, maintain the BBB and participate in the inflammatory process. Possibly the inhibition of GFAP synthesis during inflammation may delay astrogliosis, allowing neurones time to regenerate. As highly localised forms of inflammation in the CNS have profound and debilitating consequences particularly for the aged, it would be of benefit to consider how astrogliosis can be modulated to promote healing and functional recovery of neuronal pathways.

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